National Library
of Canada

Canadian Theses Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale
du Canada

Service des thèses canadiennes

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

NL-339 (r. 88/04) c

Canadä

UNIVERSITY OF ALBERTA

FRAMESHIFT MUTAGENESIS AND ANTIMUTAGENESIS IN YEAST

by

QI WANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL, 1990

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-64978-X

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR QI WANG

TITLE OF THESIS FRAMESHIFT MUTAGENESIS AND ANTIMUTAGENESIS IN YEAST

DEGREE MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED 1990

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed)

PERMANENT ADDRESS:

#1101, 11147-82 Ave
Edmonton, Alberta

DATED

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "FRAMESHIFT MUTAGENESIS AND ANTIMUTAGENESIS IN YEAST" submitted by Qi Wang in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in the Department of Genetics.

R.C. von Borstel
....
R.C. von Borstel (Supervisor)

....
P.J. Hastings

....
J.M. Tyler

....
R.S. Day III

DATE 30 August 1990

Dedicated to my parents and Lin Hui,
for their love and encouragement.

ABSTRACT

The study of the mechanisms of action of antimutagenic agents can be useful in understanding the nature and origin of spontaneous mutations. Certain chemicals exhibit the property of reducing the spontaneous mutation rates in the yeast *Saccharomyces cerevisiae*. In systems for forward and reverse mutations, 9-aminoacridine is antimutagenic during mitosis. The expression of the antimutagenic effect appears to be related to a delay in cell division.

By measuring the reversion rates of growing cells of yeast with the compartmentalization test, it has been found that the antimutagenic effect of 9-aminoacridine has allele specificity and is dose dependent. 9-Aminoacridine affects the reversion rates of the frameshift alleles *hom3-10*, *his4-38*, and *leu2-3* of strain XV195-6B, but not the base substitution alleles *arg4-17* and *his1-7* of strain XV185-14C. At higher concentrations, 9-aminoacridine delays cell division and is antimutagenic, decreasing the reversion rates of the frameshift alleles by 2-fold. At lower concentrations, 9-aminoacridine is mutagenic, increasing the reversion rates of the frameshift alleles by 4-fold. Acriflavine and acridine orange are mutagenic in yeast but do not exhibit antimutagenic properties.

The *hom3-10* mutant was used in studying frameshift mutagenesis and antimutagenesis. The *hom3-10* mutant was postulated to be a frameshift because of significant genetic evidence. It shows a strong meiotic effect, and it can be reverted by frameshift mutagens. The molecular structure of the frameshift mutation in *hom3-10* was established through studies involving the amplification of the *hom3-10* gene by the polymerase chain reaction, and double-stranded DNA sequencing. The mutation is a +1 C insertion into a proline codon. This insertion shifts the reading frame and abolishes the postulated stop codon; therefore, the resulting transcript will encode 120 amino acids more than the wild type. In strain XV195-6B, *his4-38*, *hom3-10*, and *leu2-3* all can be suppressed simultaneously. The mutation in *hom3-10* is a +1 C insertion in a proline codon, whereas the mutations in *his4-38* and *leu2-3* are +1 G insertions in glycine codons. The mechanism by which a 4-base prolyl-tRNA suppresses these three mutations simultaneously probably involves suppression at a target site other than the actual mutation site.

Acknowledgements

It is a pleasure to express my deep appreciation to my supervisor, Dr. R.C. von Borstel. He was always willing to listen to my ideas and taught me to think scientifically.

I would like to thank Dr. Ursula G.G. Hennig for her patient guidance, dedicated supervision, and constant encouragement throughout my work, and especially for her critical comments as I was writing this thesis. Thank a million, Ursula!

I thank everyone in Dr. von Borstel's laboratory for their help and making my research work a pleasant experience. I am grateful to Dr. Elizabeth A. Savage for her help in dissecting tetrads for the suppression efficiency analysis, and to Mr. R. Gary Ritzel for his valuable techanical advice. I also thank Dr. Ursula G.G. Hennig and Ms. Karla S. Blonsky for providing me with the triply suppressed revertants for suppression analysis. My gratitude is extended to Dr. Grace Lee, Ms. Diane Lee Van On, Mr. Ken Parker, and Ms. Marlice Schmidt for their help at various times.

I thank Dr. P.J. Hastings for his helpful advice. Special thanks go to Ms. Laura Querengesser who taught me how to use the Macintosh computer. I also would like to express my appreciation to Ms. Audrey Atkin for her valuable suggestions on the frameshift suppression.

TABLE OF CONTENTS

Page

INTRODUCTION 1

MATERIALS AND METHODS 17

A. Measuring spontaneous mutation rates 18
  1. Compartmentalization test 18
  2. Concentrations of limiting amino acids for the *hom3-10* locus 20
  3. Mutation rate 21

B. Measuring induced mutation rates 21
  1. 9-Aminoacridine, acriflavine, and acridine orange 21
  2. Cell generation time and viability 22
  3. Mutation rate 24

C. Molecular analysis of the *hom3-10* allele 24
  1. Extraction of genomic DNA 24
  2. Amplification of the *hom3-10* gene by the polymerase chain reaction (PCR) 26
  3. Extraction and purification of amplified DNA 28
  4. Cloning 29
  5. Transformation 32
  6. Minipreps 34
  7. DNA sequencing of the *hom3-10* gene 36

RESULTS 48

A. The limiting amino acid for the *hom3-10* locus 49

B. Reversion studies with 9-aminoacridine, acriflavine, and acridine orange 49
1. Effects on cell generation time and viability 49
2. Effect on mutation rate 50

C. Molecular studies on the *hom3-10* allele 52
1. PCR and cloning 52
2. Sequence analysis 54

DISCUSSION 69

A. Mutagenicity of acridines 70

B. Antimutagenicity of 9-aminoacridine 74

C. Suppression of frameshift mutations 79

BIBLIOGRAPHY 90

LIST OF TABLES

Table	Description	Page
1	The limiting media used for the compartmentalization tests	42
2	Acridines and acridine salts used in the compartmentalization tests	43
3	The concentrations of limiting amino acids tested for the *hom3-10* allele, as determined in strain XV185-14C	56
4	Cell generation time and viability with increasing concentrations of 9-aminoacridine, as determined in strain XV195-6B	57
5	Cell generation time and viability with increasing concentrations of acriflavine and acridine orange, as determined in strain XV195-6B	58
6	Reversion rates (mutational events/cell/generation) for two base-substitution alleles with increasing concentrations of 9-aminoacridine, as determined in strain XV185-14C	59
7	Reversion rates (mutational events/cell/generation) for several frameshift alleles with increasing concentrations of 9-aminoacridine, as determined in strain XV195-6B	60
8	Reversion rates (mutational events/cell/generation) with increasing concentrations of acriflavine and acridine orange	61
9	Misincorporation of bases by *Taq*I DNA polymerase during PCR	62

LIST OF FIGURES

Figure	Description	Page
1	The wild-type sequence of the *HOM3* gene	45
2	The pT7T3 19U cloning vector	47
3	Cell generation time and viability in strain XV195-6B with increasing concentrations of 9-aminoacridine	63
4	Cell generation time and viability in strain XV195-6B with increasing concentrations of acriflavine	64
5	Cell generation time and viability in strain XV195-6B with increasing concentrations of acridine orange	65
6	Reversion rates for *hom3-10* in strain XV195-6B with increasing concentrations of 9-aminoacridine	66
7	The *hom3-10* gene, amplified by PCR	67
8	Restriction analysis of the cloned fragments of the *hom3-10* gene	68
9	The frameshift mutation in *hom3-10* and the consequence of the mutation	87
10	Suppression of the frameshift mutations in *hom3-10*, *his4-38*, and *leu2-3*	89

INTRODUCTION

Spontaneous mutations can be defined as mutations which occur in an organism living in its normal environment. Great effort has been made in understanding this fundamental biological phenomenon of the nature and origin of spontaneous mutations.

The first molecular model for the origin of spontaneous mutations was proposed by Watson and Crick (1953). Purines and pyrimidines can exist in different tautomeric forms, of which the ones that normally pair in DNA are the major forms. The equilibrium with other minor forms (enol and imino), however, gives a certain probability that at the time of DNA replication one of the bases in the preexisting strand, or in the nucleotide pool being used for the synthesis, undergoes a tautomeric shift of a hydrogen atom and consequently makes a pairing mistake. It is very difficult and probably impossible to produce direct evidence on the validity of this model, but it has been the basis for all the subsequent work on molecular mutagenesis.

The research has continued up to the present time, with results which have given useful indications as to the origin of spontaneous mutations. There are two important approaches in the studies on the origin and nature of spontaneous mutations; one is the study of mutator and antimutator genes, and the other is the study of the mechanisms of action of antimutagenic agents.

The investigation of mutator and antimutator genes in bacteria and their phages, in comparison with the yeast *Saccharomyces cerevisiae*, has revealed that the origins of spontaneous mutations might be quite different in different organisms. The spontaneous mutations in *Escherichia coli* and its phage T4 arise by mistakes made by a polymerase-exonuclease complex during DNA replication (Speyer, 1965; Speyer *et al.*, 1966; Drake and Allen, 1968; Hershfield and Nossal, 1973). DNA polymerase has, in addition to its synthetic capacity, the ability to hydrolyze deoxynucleotide chains. The ratios of this exonuclease activity to polymerase activity, measured in arbitrary units, are different for the antimutator, mutator, and wild-type enzymes. Thus, the enzyme induced by antimutators shows a higher 3'-exonuclease to polymerase ratio, and the enzyme induced by mutators shows a lower ratio, than does the wild-type enzyme. The 3'-exonuclease seems to have an editing function, scanning newly synthesized DNA for errors made by the polymerase, and variation in the amount of editing by the exonuclease is related to the frequency of spontaneous mutations. In the analysis of mutants selected for mutator activity in the yeast *Saccharomyces cerevisiae*, the inadequacy of the editing hypothesis has become evident (Hastings *et al.*, 1976). A study of the accumulated data on radiation-sensitive mutants and mutator mutants in yeast reveals that spontaneous lesions susceptible to mutagenic repair are occurring in yeast. These same radiation-sensitive mutations affect the frequency of spontaneous mutations. This is the evidence that spontaneous lesions occur and are repaired by systems which have many steps in common with the systems that repair induced lesions. It also can be concluded that mutagenic repair is available to the spontaneous lesions, and mutators enhance spontaneous mutation rates by channelling spontaneous lesions into mutagenic repair pathways.

The study of mechanisms of action of antimutagenic agents also can be very useful for distinguishing among the different origins of spontaneous mutations. Antimutagenesis is the lowering of spontaneous mutation rates and induced mutation frequencies by exogenous conditions. The field has been divided into two parts, desmutagenesis and antimutagenesis, by Kada (1982). Desmutagenesis refers to the inactivation of mutagens and carcinogens by means such as antioxidation, cytochrome P-450-associated metabolism, or by the active destruction or absorption of the toxic substances before they can enter the target cell. Antimutagenesis is the enhancement of the nonmutagenic repair of DNA, the inactivation of mutagenic repair, or some interference with the expression of mutations.

Acridines, and a very large number of acridine derivatives, have long been employed as tools in mutation research. Acridine compounds and their derivatives form complexes with DNA and thus have a variety of biological effects. It is known that some acridines are effective mutagens in phages (DeMars, 1953; Orgel and Brenner, 1961) and in yeast during meiosis (Magni *et al.*, 1964). The mutations induced by the acridines in phage T2 and in the rII region of phage T4 are attributable to either base deletions or additions (frameshifts), and their origin has been interpreted as being related to acridine intercalation between bases in the DNA (Lerman, 1963). There are two types of interactions between acridines and double-stranded DNA, and these are defined by strong and weak binding characteristics (Peacocke and Skerrett, 1956). Lerman (1961) proposed that strong binding occurs by intercalation of acridines between adjacent base pairs. Pritchard *et al.* (1966) suggested that acridines intercalate between single adjacent (noncomplementary) bases. The weak binding occurs with the outside of the double helix, by electrostatic forces between the positively charged acridines and the negatively charged phosphate (Peacocke and Skerrett, 1956). The discovery of the intercalating action of acridines facilitated the understanding of the molecular basis of acridine mutagenesis. The observation that some acridines are also antimutagenic makes their underlying mechanism of action even more intriguing.

9-Aminoacridine has been reported to be a frameshift mutagen in bacteria and in yeast. Newton *et al.* (1972) reported that 9-aminoacridine induced frameshift mutations in the *lac* operon of *Escherichia coli*. Brown *et al.* (1980) reported that 9-aminoacridine is a strong frameshift mutagen in *Salmonella typhimurium*. Magni *et al.* (1964) showed that, in the yeast *Saccharomyces cerevisiae*, 9-aminoacridine is mutagenic during meiosis and antimutagenic during mitosis. In the canavanine sensitivity to resistance forward mutation system, during meiotic growth of the diploid strain 5300, 9-aminoacridine (at a concentration of 4 µg/ml) increased the mutation frequency by 73-fold; during mitotic growth of the haploid strain 10284, 9-aminoacridine (at a concentration of 10 µg/ml) decreased the mutation frequency by 30-fold.

Acriflavine was reported to be a mutagen in both bacteria and yeast. In *Escherichia coli*, acriflavine induced the forward mutation to phage T2 resistance (Witkin, 1947) and induced reversion to streptomycin sensitivity (Demerec *et al.*, 1951). In *Saccharomyces cerevisiae*, acriflavine was shown to induce mitochondrial mutations (respiratory-deficient "petite" mutants) specifically

(Adachi, 1969; Avers and Dryfuss, 1965; Bien and Konrad, 1972; Mahler, 1973). The studies of the mechanism of this mutagenesis showed that mitochondrial DNA and RNA synthesis are very sensitive to acriflavine when compared with nuclear DNA and RNA synthesis, and it was suggested that acriflavine may compete with repressors or cause a change in the binding of repressors to the mitochondrial DNA preferentially and allow for transcription of the region previously repressed (Meyer *et al.*, 1972).

Acridine orange was shown to be antimutagenic in bacteria, but mutagenic in yeast. Acridine orange is an antimutagen in continuous cultures of *Escherichia coli* in the absence of light (Webb and Kubitschek, 1963); at a concentration of 2.5×10^{-6} M, acridine orange decreased the mutation rate for resistance to bacteriophage T5 by 3-fold. In the yeast *Saccharomyces cerevisiae*, acridine orange induced forward and reverse mutations and it caused gene conversion specifically (Davies *et al.*, 1975; Fahrig, 1970).

Much of the progress in mutation research has depended on genetic analyses, and, for this purpose, yeast is an ideal organism. Yeast has a true nucleus, 17 chromosomes (Mortimer and Schild, 1981), introns, mitochondria, and because of the haploid and diploid cell cycles, yeast can be representative of both prokaryotes and higher eukaryotes in many respects. Also, the diploid yeast cell can undergo meiosis, which is an additional advantage in the study of mutations.

The system used to measure mutation rates is the compartmentalization test (von Borstel *et al.*, 1971). This is the most accurate method for measuring the spontaneous mutation rates of mitotic cells of yeast. The method is based on the continued growth of revertants (prototrophs) after a required nutrient in the medium has been exhausted. A large number (about 500) of small cultures (about 1 ml each) are started, and the fraction of cultures in which one or more mutations occur is measured. The P_0 method for calculating mutation rates, based on a theory by Luria and Delbrück (1943), was developed by von Borstel *et al.* (1971). The principal advantage of the P_0 method is that the results are not affected by many types of selection. That is, mutational events are counted, not mutants. For example, mutants which grow either quickly or slowly do not affect the measurement.

In order to detect many different types of mutational lesions, mutant alleles of various genes in several metabolic pathways were assembled in strain XV185-14C of *Saccharomyces cerevisiae* (constructed by S-K. Quah and R.C. von Borstel; cited in von Borstel, 1981). Strain XV185-14C has the genotype *MAT*a *ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48*.

ADE2 encodes phosphoribosylaminoimidazole carboxylase which catalyzes the sixth step of purine synthesis; the mutation in the *ADE2* gene causes the accumulation of the intermediate, phosphoribosylaminoimidazole, which polymerizes to form a red pigment that turns the cells pink and eventually red (Smirnov *et al.*, 1967). *ARG4* encodes L-argininosuccinate lyase, which is an enzyme involved in the last step of the arginine pathway; the enzyme cleaves L-argininosuccinate to produce L-arginine. *HIS1* encodes ATP-phosphoribosyltransferase, which catalyzes the first reaction in the histidine pathway; the step catalyzed is the condensation of the ribosylphosphate moiety of phosphoribosylpyrophosphate onto ATP to yield phosphoribosyl-ATP. *LYS1* encodes saccharopine dehydrogenase, which catalyzes the conversion of saccharopine into L-lysine in the last step of the lysine pathway. *TRP5* encodes tryptophan synthase, which catalyzes the cleavage of indoleglycerolphosphate and the condensation of indole with L-serine to yield L-tryptophan. *HOM3* encodes aspartokinase. In yeast, the first step in the conversion of L-aspartate to methionine and threonine is the aspartokinase-catalyzed activation of aspartate by ATP to produce β-aspartylphosphate. Therefore, a mutation in the *HOM3* gene results in a requirement for both threonine and methionine for growth.

The genetic features of the mutant alleles of strain XV185-14C are as follows: *ade2-1*, *arg4-17*, *lys1-1*, and *trp5-48* are suppressible, chain-terminating mutants, *his1-7* is a missense mutant that is not suppressible by external suppressors, and *hom3-10* is a frameshift mutant.

The chain-terminating mutants are of the ocher (UAA) type (Gilmore *et al.*, 1971; Gesteland *et al.*, 1976). The ocher nonsense suppressor, *SUP4*-o, was sequenced (Goodman *et al.*, 1977) and the mutant site was found to be a thymine in place of a guanine at the wobble position of a tRNA^{Tyr}. The types of revertants of *ade2-1*, *arg4-17*, and *lys1-1* can be deduced from their phenotypes. The 5'-UAA-3' ocher nonsense codon could revert to either 5'-UAU-3' or 5'-UAC-3', both of which are tyrosine codons, indicating that reversions at the locus comprise AT->TA and AT->CG transversions (von Borstel *et al.*, 1973). The anticodon of the tRNA^{Tyr} could mutate from 5'-GΨA-3' to 5'-ZΨA-3', where Z is the unknown modified uridine and Ψ is pseudouridine (Sherman, 1982). Therefore the forward mutations in the anticodon of the $tRNA^{Tyr}$ are GC->TA transversions, resulting in an ocher-suppressing phenotype.

A few ocher nonsense mutations also can be suppressed by psi factor, which is a modifier of translational suppression in yeast (Cox, 1965). Locus reversions can be identified easily by replica plating the apparent revertants onto omission media and by noting the color (red or white) of the colonies. The allele *trp5-48* is suppressed by the psi factor, so when the psi factor is present, since it is maternally inherited, spores become unscoreable for the Trp^- phenotype (Cox *et al.*, 1988). To identify the introduction of psi factors through crosses, *trp5-48* is constructed into most strains of yeast used for mutation analysis. Although various models for the inheritance and activity of the psi factor have been proposed (Cox *et al.*, 1988), the mechanism by which the psi factor causes suppression remains elusive.

The *his1-7* mutant has a spontaneous reversion rate that is similar to the forward spontaneous mutation rate from canavanine sensitivity to resistance (Gottlieb and von Borstel, 1976). When revertants of *his1-7* were characterized, it was found that most of the reversions occurred by second-site mutations in the *HIS1* locus (Lax and Fogel, 1978). The reversion of *his1-7* by intragenic missense suppression is also evident from the phenotypic heterogeneity of the revertants, since many of them have been observed to be feeder colonies (Meadows, 1973; Hennig, 1984). The *his1-7* mutant is osmotically remediable by 1 M KCl when grown at

18°C (Hawthorne and Friis, 1964), which suggests that it is a missense mutant. Reversion studies have shown that *his1-7* is highly revertible with ethyl methanesulfonate (Shahin and von Borstel, 1978), which primarily induces transitions in phage T4 (Krieg, 1963) and in *Neurospora crassa* (Malling and de Serres, 1968). Also, *his1-7* is reverted by the mutator *mut7,8*, which appears to induce preferentially AT->TA and AT->CG transversions (D.S. Bendiak and J.M. Tyler, unpublished data). The evidence indicates that both transitions and transversions can revert the *his1-7* mutation, although the largest proportion of the revertants would be transitions. The molecular characterization, by DNA sequencing, of *his1-7* has shown that the mutation is a GC -> AT transition in a methionine codon (E.A. Savage and R.G. Ritzel, unpublished data). This mutation changes the methionine codon (AUG) to an isoleucine codon (AUA).

The *hom3-10* mutant was postulated to be a frameshift because of significant genetic evidence. The *hom3-10* allele shows a strong meiotic effect (Magni and Puglisi, 1966). The meiotic effect on spontaneous mutations in yeast was discovered by Magni and von Borstel (1962). In several mutants carried in homozygous conditions by diploid cells, this effect has been described as a spontaneous reversion rate that is 10 - 30 times higher during meiosis than in mitosis. Chromosome pairing in the region where mutations occur is a prerequisite for the increase in the spontaneous reversion rate during meiosis. This was indicated by the fact that the meiotic effect on the reversion of the mutant *thr4-1* was abolished completely when one of the two homologous chromosomes carried a long deletion covering the *thr4* locus (Magni, 1963). Furthermore, outside marker recombination concomitant with the meiotic reversion of the *his1-1* mutant showed that a large fraction of meiotic mutants are associated with crossing over in the region where the mutational event takes place (Magni, 1963). The evidence, when compiled, supports the model proposed by Magni (1963), which suggests that spontaneous mutations in meiosis arise as a consequence of unequal crossing over, causing the insertion or deletion of one or more bases. Mutants showing an increased rate of reversion in meiosis should be of the frameshift type, and the meiotic effect should be completely absent in the reversion of base substitution mutations. The *hom3-10* allele shows a very strong meiotic effect; the reversion rate for *hom3-10*->*HOM*$^+$ is 27.5 times higher during meiosis than the rate during mitosis (Magni and Puglisi, 1966). Therefore, it is believed that the reversions of *hom3-10* are frameshifts.

The reversion of *hom3-10* can be induced by frameshift mutagens. Hycanthone, which is an antischistosomal drug, was shown to be a frameshift mutagen in *Salmonella typhimurium* (Hartman *et al.*, 1971, 1973). When the mutagenicity of hycanthone was tested in yeast, it was found that the drug increased the reversion frequency of *hom3-10* dramatically (Meadows *et al.*, 1973). This suggests that the reversion of *hom3-10* occurs by base addition or deletion. Pyrvinium pamoate, an anthelmintic drug, was characterized by measuring its ability to unwind supercoiled DNA and to lengthen circular duplex DNA molecules; the results led to the conclusion that the primary interaction of pyrvinium compounds with duplex DNA involves intercalation (Dickie *et al.*, 1985). The intercalating action of pyrvinium pamoate is a characteristic of frameshift mutagens, and this frameshift mutagenicity was detected in bacteria and in yeast. When tested in *Salmonella typhimurium*, it was shown that pyrvinium pamoate is mutagenic with the frameshift tester strains TA97 and TA98 (Hennig *et al.*, 1987). In the yeast strains XY718-1A and 7854-1A, the frameshift alleles *his4-38, his4-519,* and *leu2-3* are highly revertible with pyrvinium pamoate (Hennig *et al.*, 1987). In the same study, it was also found that pyrvinium pamoate has the same effect on the reversion of *hom3-10* as it has on *his4-38*, *his4-519*, and *leu2-3*. This suggested that *hom3-10* is a frameshift mutant.

For the specific study of frameshift mutations, strain XV195-6B of *Saccharomyces cerevisiae* was constructed (von Borstel *et al.*, 1986). This strain has the genotype *MAT*a *ade2-1 arg4-17 his4-38 hom3-10 leu2-3 lys1-1*. XV195-6B was constructed by crossing strain XV185-6B with strain 7854-1A, which has the genotype *MAT*a *his4-38 leu2-3*. XV185-6B and XV185-14C come from the same parents and have identical genotypes, except that they have opposite mating types.

The *HIS4* gene encodes a single, trifunctional protein which is involved in three steps in the pathway of histidine biosynthesis. The polypeptide is divided into three domains of function and each of these domains is encoded by a subregion of *HIS4* (Keesey *et al.*, 1979; Fink, 1964). The domains of the protein and the subregions of the gene are colinear with one another and the order of the subregions within the gene is *HIS4A, B,* and *C*. Region *A* (*HIS4A*) encodes phosphoribosyl-AMP cyclohydrolase, which catalyzes the conversion of 1-*N*-(5'-phosphoribosyl)-AMP to phosphoribosyl-formimino-5-aminoimidazolecarboxamide ribonucleotide. Region *B* (*HIS4B*) encodes phosphoribosyl-ATP pyrophosphohydrolase, which catalyzes the pyrophosphorolysis of 1-*N*-(5'-phosphoribosyl)-ATP to yield 1-*N*-(5'-phosphoribosyl)-AMP. Region *C* (*HIS4C*) encodes histidinol dehydrogenase, which catalyzes the oxidization of histidinol to yield L-histidine. The *LEU2* gene encodes β-isopropylmalate dehydrogenase, which is the third enzyme in leucine biosynthesis; this enzyme catalyzes the oxidative decarboxylation of β-isopropylmalate to yield α-ketoisocaproate, the precursor of L-leucine.

Both *his4-38* and *leu2-3* were induced by the frameshift mutagen ICR 170 (Culbertson *et al.*, 1977) and both mutants have a +1 base (G) insertion in a glycine codon (Donahue *et al.*, 1981; Edelman, 1987). The *his4-38* and *leu2-3* mutants can be suppressed by frameshift suppressors (Culbertson *et al.*, 1977), which are mutant tRNAs that recognize 4-base glycine codons. Frameshift suppressing tRNAs result from a forward mutation (a +1 base insertion) in the anticodon of the tRNA, and these mutant tRNAs insert an amino acid in response to a 4-base codon in the mRNA, thus maintaining the correct reading frame. Neither *his4-38* nor *leu2-3* can be suppressed by nonsense suppressors (Fink and Styles, 1974), and both alleles revert at high frequencies with ICR 170 and at low frequencies with ethyl methanesulfonate (Culbertson *et al.*, 1977). Mutagenicity studies have shown that *his4-38* and *leu2-3* are highly revertible by the frameshift mutagen pyrvinium pamoate (Hennig *et* *al.*, 1987). The frameshift suppression, which can be identified by coreversion analysis (apparent revertants with a His^+Leu^+ phenotype), has been observed in several studies (Culbertson *et al.*, 1977, 1980; Hennig *et al.*, 1987; P.O. Andersson, E.A. Savage, and U.G.G. Hennig, unpublished data).

Statement of the problem

It has been established that many acridine compounds and their derivatives are mutagens. At the molecular level, acridines cause frameshift mutations specifically in a variety of organisms, and the origin of these mutations has been interpreted as being related to the intercalation of acridines between bases in the DNA (Lerman, 1963). The observation that some acridines exhibit an antimutagenic effect makes their underlying mechanisms of action even more intriguing. More important, to investigate the mechanisms of action of antimutagens would be a valuable approach in studying the origins of spontaneous mutations.

9-Aminoacridine has been reported to be an antimutagen in yeast during mitosis (Magni *et al.*, 1964). In order to understand the influence of 9-aminoacridine on the yields of spontaneous mutations, it is essential to study 9-aminoacridine more closely for its molecular action. The functional groups on the acridine ring system may influence the mutagenic and antimutagenic effects of acridines such as acriflavine and acridine orange. These structure-activity relationships can be examined with analogs or derivatives of the acridine ring systems.

The yeast strain XV185-14C can be used for studying the mutation spectrum since it contains a variety of mutant alleles. By studying the effect of 9-aminoacridine on the reversion rates of the different alleles in XV185-14C, any allele specificity that the acridines may have in mutagenesis and antimutagenesis could be identified. An analysis of the processes which may be involved in generating the spontaneous frameshift and base substitution mutations could be made.

It also has been reported that a concentration of 9-aminoacridine that causes a 2- or 3-fold delay in the cell generation time is required to observe a significant antimutagenic effect (Puglisi, 1966), suggesting that the antimutagenic effect could be related to the delay in cell division. By comparing the effects of several acridines on cell division, the relationship between the delay in cell division and antimutagenesis could be investigated.

The *hom3-10* allele in strain XV185-14C is believed to be a frameshift mutant because of significant genetic evidence. The *hom3-10* mutant shows a strong meiotic effect (Magni *et al.*, 1966), and it can be reverted by frameshift mutagens (Meadows *et al.*, 1973; Hennig *et al.*, 1987). It is important to provide some molecular evidence for the characterization of *hom3-10*. By sequencing the mutant *hom3-10* gene, and comparing the sequence with that of the published sequence for the wild-type *HOM3* gene (Rafalski and Falco, 1988), it should be possible to identify the mutation.

The yeast strain XV195-6B had been constructed for studying frameshift mutations specifically (von Borstel *et al.*, 1986). The three frameshift alleles of this strain are *his4-38*, *hom3-10*, and *leu2-3*. The mutations in *his4-38* and *leu2-3* are +1 G insertions in glycine codons (Donahue *et al.*, 1981; Edelman, 1987). Corevertants (apparent revertants with a His$^+$Leu$^+$ phenotype) have been observed in several studies (Culbertson *et al.*, 1977; Hennig *et al.*, 1987; P.O. Andersson, E.A. Savage, and U.G.G. Hennig, unpublished data), and these are attributed to frameshift suppression. Apparent revertants with a Hom$^+$His$^+$Leu$^+$ phenotype also have been observed (U.G.G. Hennig and K.S. Blonsky, unpublished data). With DNA sequence data for the *hom3-10* mutation, the mechanism for this frameshift suppression can be studied further.

MATERIALS AND METHODS

A. Measuring spontaneous mutation rates

1. Compartmentalization test

a. Strains

The strains of the yeast *Saccharomyces cerevisiae* that were used are XV185-14C and XV195-6B. The genotype of strain XV185-14C is *MAT*a *ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48*, and the genotype of strain XV195-6B is *MAT*a *ade2-1 arg4-17 his4-38 hom3-10 leu2-3 lys1-1*. The genetic markers of these strains were checked routinely.

b. Media

The concentrations for the stock solutions of amino acids and bases were 2 g/L for adenine, L-arginine, L-histidine, L-methionine, L-tryptophan, and uracil; 3 g/L for L-lysine; and 5 g/L for L-leucine and L-threonine. These stock solutions were prepared in volumetric flasks. Uracil was autoclaved at 121°C for 20 min, whereas the other stock solutions were filter-sterilized. L-Tryptophan, which is heat labile and sensitive to light, was stored in the dark.

YEPD (1% yeast extract, 2% peptone, and 2% dextrose) is a complete medium which is used for routine growth and viability assays; for a solid medium, 1.5% agar was added to prepare YEPD plates. Liquid synthetic complete (SC) medium consists of minimal medium (0.67% yeast nitrogen base without amino acids and 2% dextrose) plus the amino acids and bases required for strains XV185-14C and XV195-6B (20 mg/L for each of adenine, arginine, histidine, methionine, tryptophan, and uracil; 30 mg/L of lysine; 50 mg/L for each of leucine and threonine). For a solid medium, 2% agar was added to liquid SC medium to prepare SC plates.

For the compartmentalization tests, a limiting medium was used. Limiting medium is liquid SC medium with one of the nutrilites at a concentration low enough to limit the growth of the yeast cells. Different limiting media were used for the various alleles of strains XV185-14C and XV195-6B (Table 1).

An omission medium is SC medium from which one of the required supplements has been omitted (*e.g.*, SC-his and SC-thr). Solid media were prepared by adding 2% agar to minimal medium, liquid SC medium, or liquid SC omission media. For each type of medium, the dextrose solution was always autoclaved separately at 121°C for 20 min and added to the other ingredients after sterilization; the amino acids and bases were added last and the medium was brought up to volume with hot sterile water.

c. Compartmentalization test

The yeast strains were grown on YEPD plates at 30°C. A single pink colony was suspended in 5 ml of sterile water, the cell concentration was determined with a hemocytometer, and the suspension was adjusted to 5×10^6 cells/ml with sterile water. A 0.25-ml aliquot (1.25×10^6 cells) of the suspension was added to 600 ml of limiting medium. Thus the limiting medium was inoculated with 2000 - 5000 cells/ml, and 1-ml aliquots of this inoculum were then distributed into 100-compartment cell culture trays (Falcon Plastics) with a Brewer automatic pipetting machine. For the control (spontaneous mutation rate) experiments, the automatic pipetting machine was used; however, when a toxic chemical was added to the medium, the 1-ml aliquots were distributed with disposable 25-ml plastic pipettes. For each experiment, 500 compartments (five 100-compartment cell culture trays) were used. After the suspension had been distributed into the compartments, the trays were wrapped with plastic bags or plastic wrap to minimize evaporation; the cells were incubated at 26°C, in the dark, for 12 days. In the limiting medium, the yeast cells grow to saturation within 24 - 48 hours; after 2 days, only the reverted cells keep growing and the revertant colonies begin to appear after 3 days.

To estimate the background mutation frequency, 0.5 ml of the cell suspension (5×10^6 cells/ml) was spread onto each of two plates of the appropriate omission medium (SC minus the limiting amino acid). In order to determine the number of viable yeast cells in the inoculum, 1 ml of the inoculated limiting medium was diluted with 9 ml of sterile water and 0.5 ml of this dilution was spread onto each of two YEPD plates.

2. Concentrations of limiting amino acids for the *hom3-10* locus

Small scale (100 compartments) mutation rate experiments were used to determine the optimum limiting concentrations of threonine and methionine for the *hom3-10* locus in strains XV185-14C and XV195-6B. Several concentrations of threonine (1.0, 1.5, 2.0, 2.5, and 3 mg/L) and methionine (0.1, 0.3, 0.5, 0.7, 0.9, and 1.1 mg/L) were tested to find the optimum limiting concentration for each of these amino acids. There are two major criteria for choosing the optimum limiting concentration of an amino acid. First, there should not be a continuous reversion, which means that after 12 days of incubation new revertant colonies do not appear. Second, the number of cells should be high enough in each of the compartments to allow the revertant colonies to appear.

3. Mutation rate

Using the P_0 method, which involves counting the number of compartments without revertants and knowing the number of cells in the mutant-free compartments, the mutation rate can be computed (von Borstel, 1978).

N_o is the number of compartments without revertants; $N_o = N_t - N_r - N_c$, where N_t is the total number of compartments filled, N_r is the number of compartments containing one or more revertant colonies, and N_c is the number of contaminated compartments. N is the number of compartments without contamination ($N = N_t - N_c$).

$N_o/N = e^{-m}$, and $\ln e^{-m} = \ln N_o/N = -m$, where m is the average number of mutational events per compartment.

The mutation rate, M, is mutational events per cell per generation during the growth period in the compartments. $M = \frac{m - m_b}{2C}$, where m_b is the number of background mutants per compartment in the inoculum (as determined by direct plating) and C is the average number of cells per compartment.

B. Measuring induced mutation rates

1. 9-Aminoacridine, acriflavine, and acridine orange

The 1 mM stock solution of 9-aminoacridine was prepared by dissolving 3.9 mg of 9-aminoacridine hydrochloride (Lot# 105F-0528, Sigma Chemical Co.) in 20 ml of water. The 0.5 mM stock solution of acriflavine (Lot# 6507, Nutritional Biochemicals Corporation) was prepared by dissolving 2.26 mg of acriflavine (a mixture of 3,6-diamino-10-methylacridinium chloride and 3,6-diaminoacridine in 20 ml of water. The 0.5 mM stock solution of acridine orange (Lot# 96F-3668, Sigma Chemical Co.) was prepared by dissolving 2.65 mg of acridine orange base (85% dye content) in 20 ml of water. All of these stock solutions were stirred until the acridine had dissolved, after which they were filter-sterilized. The stock solutions were stored at 4°C, in the dark. Details on the acridine compounds are described in Table 2.

2. Cell generation time and viability

The cell generation time was measured in complete medium and in limiting medium.

a. In complete medium

Each strain of yeast was grown on a YEPD plate at 30°C and a single pink colony was used to start an overnight culture in 5 ml of liquid YEPD; the culture was incubated at 30°C in an incubator shaker at 125 rpm. The next morning, 1 ml of overnight culture was diluted with 24 ml of liquid YEPD medium in a sterile 250-ml flask. From this 25 ml of diluted overnight culture, 1 ml was diluted with sterile water and the cell number was counted, under the microscope, with a hemocytometer; this was the cell concentration at time zero (t = 0). The remaining 24 ml of culture were incubated continuously at 30°C and at t = 1, 2, 3, 4, 5, and 6 hours, 1-ml aliquots of culture were removed and diluted with sterile water to determine the cell concentrations at 1-hour intervals until the culture became saturated (at t = 6).

Whenever the cell number was counted the viability was also determined. This was done by spreading 0.5 ml of a dilution which contained 200 - 300 cells/ml onto each of four YEPD plates. The plates were incubated at 30°C for 3 days and the number of colonies were counted. The viability is defined as the percentage of cells, which had been observed under the microscope, that were able to divide and form colonies. The results from the viability tests were used to calculate the cell generation time.

b. In limiting medium

This method was used to determine the effect of several acridines on the cell generation time and the results were compared with the generation time of the untreated strain (control experiments) in liquid YEPD and limiting medium.

Each strain of yeast was grown on a YEPD plate at 30°C and a single pink colony was suspended in 2 ml of sterile water. The cell number was counted with a hemocytometer, the suspension was adjusted to 2×10^8 cells/ml with sterile water, and 0.1 ml (2×10^7 cells) of this adjusted suspension was added to 100 ml of limiting medium which contained 9-aminoacridine, acriflavine, or acridine orange. The inoculum containing the acridine was distributed in 1-ml aliquots (2×10^5 cells) into 100 compartments and the cell culture trays were incubated continuously at 30°C, in the dark. The cell number was counted, under the microscope with a hemocytometer, at t = 0, 1, 2, 3, 4, 5, and 6 hours, when the culture became saturated. At each time interval, the viability was determined by spreading the appropriate dilution onto YEPD plates. The cell viability and generation times were determined in media with limiting arginine, histidine (for *his1-7*), or methionine (Table 1).

3. Mutation rate

The compartmentalization test was used to determine the mutation rates induced by 9-aminoacridine, acriflavine, and acridine orange. For each acridine, the concentrations which did not affect the cell generation time, the concentrations which delayed the cell generation time without a concurrent decrease in viability, and the concentrations which delayed the cell generation time with a concurrent decrease in viability were chosen for the compartmentalization test. By adding the appropriate amount of the stock solution of an acridine into the limiting medium, the desired final concentration was obtained. As a control, the spontaneous mutation rate was measured simultaneously in each set of experiments. The cell generation time and viability were determined during the 12 days of incubation by counting the cell number and plating an appropriate dilution of the sample each day.

C. Molecular analysis of the *hom3-10* allele

1. Extraction of genomic DNA

The genomic DNA of strains XV185-14C and XV195-6B was extracted using a modification of the procedure of Sherman *et al.* (1986). A single pink colony, from a YEPD plate which had been incubated at 30°C, was suspended in 40 ml of liquid YEPD medium in a 125-ml flask. The culture was grown to saturation in an incubator shaker (125 rpm) at 30°C. The cells in the saturated culture were pelleted (Sorvall RC-5B centrifuge at 5000 rpm for 5 min), the supernatant was discarded, and the pellet of cells was resuspended in 5 ml of 1.2 M sorbitol. This suspension of cells was then pelleted, the supernatant was discarded, the pellet of cells was resuspended in 3 ml of freshly prepared zymolyase solution (0.9 M sorbitol, 0.1 M EDTA, 1.7 mM β-mercaptoethanol, and 0.2% zymolyase 20,000), and incubated at 37°C for 60 min. The lysate was centrifuged at 5000 rpm for 5 min, the pellet was resuspended in 5 ml of TE pH 7.4 (50 mM TRIS-HCl and 20 mM EDTA), 0.5 ml of 10% SDS (sodium dodecyl sulfate) was added with vigorous mixing, and the preparation was incubated at 65°C for 30 min. After 1.5 ml of 5 M potassium acetate had been added, the lysate was left on ice for 60 min, centrifuged at 10,000 rpm for 10 min, the supernatant was transferred to a new plastic centrifuge tube, and 2 volumes of 95% ethanol were added to this supernatant. The preparation was mixed well at room temperature, centrifuged at 6000 rpm for 15 min, the supernatant was discarded, the pellet was air dried, and the dry pellet was resuspended in 3 ml of TE pH 7.4 and left at 4°C overnight.

The next day, this resuspended pellet was centrifuged at 10,000 rpm for 15 min, the supernatant was transferred to a new tube, 15 µl of RNaseA stock solution (10 mg/ml) were added, and the mixture was incubated at 37°C for 30 min. The RNaseA stock solution was prepared by dissolving RNaseA in a solution of 10 mM TRIS-HCl pH 7.5 and 15 mM NaCl, heating to 100°C for 15 min, and storing the aliquoted stocks at -20°C. Then 3 ml of isopropanol (99% molecular purity) were added with gentle mixing and the precipitate, which looked like a loose cocoon of fibers, was removed and suspended in 0.5 ml of TE pH 7.4. The precipitate was left at 4°C overnight to allow it time to dissolve.

The concentration of the genomic DNA was measured with an LKB spectrophotometer; the DNA sample was diluted by adding 10 µl of genomic DNA solution to 990 µl of TE pH 7.4 and from the value obtained at OD_{260nm}, the concentration of the DNA in the solution was calculated (1 OD_{260nm} = 50 µg DNA/ml). The OD_{260nm}/OD_{280nm} ratio was also calculated to determine the quality of the genomic DNA preparation. The optimum ratio is between 1.8 and 2.0. If there is contamination with protein or phenol, the ratio will be significantly lower than 1.8, whereas if there is contamination with RNA, the ratio will be significantly higher than 2.0 (Maniatis *et al.*, 1982).

2. Amplification of the *hom3-10* gene by the polymerase chain reaction (PCR)

The genomic DNA served as a template in PCR to amplify the *hom3-10* gene. Using the published wild-type *HOM3* sequence (Rafalski and Falco, 1988) as a guide, two oligonucleotides were synthesized for use as primers. Both oligonucleotides are 20-mers which span the entire coding region of the *HOM3* gene (Fig. 1). The target sequence for primer A (5'-GCCAATACTCTCTCCATCGC-3') is in the 5'-regulatory region of the gene and the target sequence for primer B (5'-TTCCTCAGCTTGTCTCAGAG-3') is in the 3' region of the gene, after the first stop codon. These primers hybridize to opposite strands of the target sequence and are oriented so that DNA synthesis by the DNA polymerase proceeds across the region between the primers. The size of the region spanned by these two primers is 1.3 kb, of which 1.2 kb is the coding region.

PCR was carried out in a Thermal Reactor TR96 (Tyler Research Instruments Corporation). The buffer (2X PCR mix) for the *Taq* DNA polymerase, which was the enzyme used to synthesize the DNA in the PCR procedure, was prepared using a modification of the protocol of Kim and Smithies (1988). The 2X PCR mix contained 130 mM TRIS-HCl pH 9.0, 20 mM $MgCl_2$, 30 mM ammonium sulfate, 10 mM β-mercaptoethanol, 1 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 5 µg/ml of each of primers A and B, and 0.2 mg/ml of bovine serum albumin. Into a 400-µl microcentrifuge tube, [illegible]0 µl of 2X PCR mix and 1.5 µg of genomic DNA were added and the final volume was brought to 40 µl with sterile water; 2.25 units of *Taq* DNA polymerase (Promega Corporation) were added and mixed well, and last, 20 µl of light paraffin oil were added to prevent evaporation.

The tubes were placed into the chamber of the thermal reactor to begin the amplification of the DNA. The program for the PCR procedure was preset in the computer attached to the thermal reactor. In the program, the initial denaturation step was 4 min at 94°C, the primer-template annealing step was 30 sec at 60°C, and the elongation step was 3 min at 73°C. Beginning with the second cycle and thereafter, the denaturing step changed to 5 min at 91°C. When a total of 30 cycles had been completed, the tubes were removed from the chamber of the thermal reactor, and without disturbing the oil layer, the aqueous lower phase was removed and transferred into a new microcentrifuge tube.

3. Extraction and purification of amplified DNA

The DNA was loaded onto a 0.9% low melting agarose gel, along with lambda DNA (*c*Iind1ts857*S*am7, Bethesda Research Laboratories, Cat# 5250SA) size markers. The gel electrophoresis was carried out at 80 volts for 3 - 4 hours and the bands were localized under ultraviolet light (300 nm). Using a sharp scalpel, a thin incision which was about 1 mm wider than the band on either side, was made in the gel directly in front of the leading edge of the band. A piece of Whatman DE81 positively charged ion exchange paper was cut to fit the width of the incision, and slightly deeper than the gel. The DE81 paper was inserted with care so that air bubbles were not trapped between the gel and the paper. Electrophoresis was continued at 40 volts until the band of DNA had migrated into the paper.

To extract the DNA, the DE81 paper was transferred into a 1-ml pipette tip, pushed to the bottom of the tip, and the remaining liquid was blown out. The pipette tip acts like a separation column; 400 µl of low salt buffer (0.2 M NaCl, 20 mM TRIS-HCl pH7.5, and 1 mM EDTA) were flushed through the tip to wash the DE81 paper and this step was repeated twice. The pipette tip containing the washed DE81 paper was placed into a 1.5-ml microcentrifuge tube, 200 µl of extra high salt buffer (1.5 M NaCl, 10 mM TRIS-HCl pH 7.5, and 1 mM EDTA) were added into the pipette tip, an incubation at 65°C for 15 min followed, and the buffer was blown into the microcentrifuge tube. This step with extra high salt buffer was repeated once and a total volume of 400 µl of DNA solution was obtained. The 400 µl of DNA solution were centrifuged at full speed for 20 min, the supernatant was transferred to a new microcentrifuge tube, and extracted twice with phenol:chloroform (7:3). The extraction step involved mixing the DNA sample with an equal volume of phenol:chloroform, centrifuging at full speed for 3 min, and transferring the aqueous upper phase to a new microcentrifuge tube; 2 volumes of 95% ethanol were added to the upper phase and the preparation was left at -20°C for 30 min. The chilled DNA preparation was centrifuged at full speed for 15 min, the supernatant was discarded, the pellet was washed once with 70% ethanol and air dried, and the dry pellet was dissolved in 40 µl of TE pH 7.5. The concentration of DNA was quantified by comparing a sample of the DNA with 0.2 µg of pBR322 DNA on a 0.8% agarose minigel.

4. Cloning

For sequencing purposes, the PCR amplified *hom3-10* gene was cloned and subcloned. The intact 1.3 kb amplified fragment, the 0.76 kb *Kpn*I fragment, and the 0.36 kb *Sau*3A fragment (Fig. 1) were cloned into a pT7T3 19U vector. The pT7T3 vectors are multi-functional plasmids that combine the advantages of both double-stranded (*e.g.*, PUC) and single-stranded (*e.g.*, M13) cloning vectors. The multi-cloning site (MCS) region is 50 bp long and contains the recognition sites for 15 different restriction enzymes (Fig. 2). Like other vectors in the PUC series, pT7T3 19U carries a short segment of *Escherichia coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lacZ*), and the MCS is embedded in this coding region. The MCS does not disrupt the reading frame but results in the harmless interpolation of a small number of amino acids into the amino terminal fragment of β-galactosidase. JM83 host cells code for the carboxy-terminal portion of β-galactosidase. Although neither the host-encoded nor the plasmid-encoded fragments are themselves active, they can associate to form an enzymatically active protein. This type of complementation is called α-complementation (Ullmann *et al.*, 1967). This is the principle for the selection of a recombinant plasmid by using this type of vector. The MCS region is flanked by a universal primer site and a reverse primer site, which are useful for DNA sequencing (Fig. 2). The pT7T3 vector used in this study was isolated from strain JM83 of *Escherichia coli* by the cesium chloride-ethidium bromide gradient method (Maniatis *et al.*, 1982).

For the cloning of the intact amplified fragment, the vector was digested with *Sma*I restriction endonuclease. The total volume of the digestion reaction was 50 µl (2 µg of pT7T3 DNA, 10 units of *Sma*I, and 5 µl of 10X Reaction 4 buffer for *Sma*I which contains 200 mM TRIS-HCl pH 8.0, 100 mM $MgCl_2$ and 500 mM KCl). For the cloning of the *Kpn*I fragment, both the vector and the PCR amplified product were digested with *Kpn*I restriction endonuclease. The total volume of the digestion reaction of the vector was 50 µl (1 µg of pT7T3 DNA, 10 units of *Kpn*I, and 5 µl of 10X Reaction 4 buffer), and the total volume of the digestion reaction of the PCR amplified fragment was 20 µl (0.5 µg of amplified fragment, 10 units of *Kpn*I, and 2 µl of 10X Reaction 4 buffer). For the cloning of the *Sau*3A fragment, the vector was digested with *Bam*HI restriction endonuclease and the

PCR amplified fragment was digested with *Sau*3A restriction endonuclease. The total volume of the digestion reaction of the vector was 50 µl (1 µg of pT7T3 DNA, 10 units of *Bam*HI, and 5 µl of 10X Reaction 3 buffer for *Bam*HI which contains 200 mM TRIS-HCl pH 7.4, 50 mM $MgCl_2$, and 500 mM KCl), and the total volume of the digestion reaction of the PCR amplified fragment was 20 µl (0.5 µg of amplified fragment, 10 units of *Sau*3A, and 2 µl of 10X Reaction 4 buffer). All of the digestion reactions were incubated at 37°C for 3 hours.

To confirm the completion of the digestions, 1/10 of each reaction was loaded onto a 0.8% agarose minigel. A completed digestion of the vector with either *Kpn*I, *Sma*I, or *Sau*3A revealed a single band of 2.9 kb. Three bands were observed upon a completed digestion of the PCR amplified fragment with either *Kpn*I (0.76 kb, 0.31 kb, and 0.25 kb) or *Sau*3A (0.48 kb, 0.46 kb, and 0.36 kb). The remaining 9/10 of the reaction were extracted once with phenol:chloroform (7:3) and once with chloroform by centrifuging at full speed for 3 min. To the aqueous upper phase (supernatant), 1/10 volume of 3 M sodium acetate and 2 volumes of 95% ethanol were added to precipitate the DNA. The precipitate was left at -20°C for 20 min, centrifuged for 20 min, and the pellet was air dried. The dry pellet was dissolved in 20 µl of TE pH 7.5 and the concentration of DNA was quantified by comparing a sample of the DNA with 0.2 µg of pBR322 DNA on a 0.8% agarose minigel.

The intact PCR amplified fragment was ligated to the pT7T3 vector, which had been digested with *Sma*I, by blunt-end ligation; the total volume of the ligation reaction was 10 µl (0.6 µg of vector

DNA, 0.3 µg of amplified fragment, 10 units of T4 ligase, and 1 µl of 10X ligation buffer which contains 0.66 M TRIS-HCl pH 7.6, 50 mM $MgCl_2$, 50 mM dithiothreitol, and 10 mM ATP). The *Kpn*I fragment of the amplified gene was ligated to the vector, which had been digested with *Kpn*I, by cohesive-end ligation; the total volume of the ligation reaction was 20 µl (0.1 µg of vector DNA, 0.3 µg of *Kpn*I fragment, 0.3 units of T4 ligase, and 2 µl of 10X ligation buffer). The *Sau*3A fragment of the amplified gene was ligated to the vector, which had been digested with *Bam*HI, by cohesive-end ligation; the total volume of the ligation reaction was 20 µl (0.1 µg of vector DNA, 0.1 µg of *Sau*3A fragment, 0.3 units of T4 ligase, and 2 µl of 10X ligation buffer). For each of the three ligation reactions, comparable experiments were set up minus the *hom3-10* fragment, as ligation controls.

5. Transformation

Competent recipient cells of strain JM83 of *Escherichia coli* were used in the transformation experiments. Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.1% dextrose, and 1% NaCl; the pH was adjusted to 7.5 with 1 M NaOH) is the growth medium for the bacteria. For a solid medium, 1.5% agar was added to the liquid LB after the pH had been adjusted to prepare LB plates. The competent cells were prepared by taking a single JM83 colony from an LB plate, which had been incubated at 37°C, to start an overnight culture in 5 ml of liquid LB; the culture was incubated at 37°C in an incubator shaker at 125 rpm. The next morning, 0.25 ml of the overnight culture was transferred into 25 ml of liquid LB medium in a 250-ml flask and the culture was incubated further (37°C at 125 rpm) until it reached OD_{600} = 0.4; 10 ml of this culture were transferred into a 15 ml tube and centrifuged at 4000 rpm for 5 min. The supernatant was discarded, the pellet of cells was resuspended in 10 ml of ice-cold transformation buffer (5 mM TRIS-HCl pH 7.5, 5 mM $MgCl_2$, and 100 mM $CaCl_2$), and the suspension was centrifuged for 10 min. The supernatant was discarded and the pellet was resuspended by repeat pipetting very gently in 1.7 ml of the transformation buffer. These competent cells may be used after 4 - 20 hours of storage at 4°C.

For the transformation experiments, 200 µl of competent cells were mixed with 2 µl of the completed *Sma*I ligation reaction and the comparable ligation control reaction, and 200 µl of competent cells were mixed with 10 µl of the other two completed ligation reactions. As a positive control, 200 µl of competent cells were mixed with 0.1 µg of uncut pT7T3 vector DNA. Also, 200 µl of competent cells were mixed with 0.1 µg of cut, non-ligated vector DNA (three controls, cut with either *Sma*I, *Kpn*I, or *Bam*HI). All of the reactions were placed on ice for 30 min and then incubated at 42°C for 90 sec (heat shock). Then, 800 µl of liquid LB were added to each reaction, and the reactions were incubated at 37°C for 45 min and spread onto LBA-Xgal-IPTG plates. These plates were prepared by dissolving 50 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) and 25 mg of isopropyl-β-D-thiogalactoside (IPTG) in 2 ml of *N,N'*-dimethylformamide (DMF) and adding this solution, plus 4 ml of sodium ampicillin stock solution (25 mg/ml), to 1 liter of LB agar just before pouring the plates. Both Xgal and IPTG are substrates for β-galactosidase. In the presence of Xgal, the Lac+ bacteria that result from α-complementation form blue colonies. However, the insertion of a fragment of foreign DNA into the MCS site of the plasmid almost invariably results in the production of an amino-terminal fragment that is not capable of α-complementation. Bacteria carrying recombinant plasmids therefore form white colonies.

As a negative control, 100 µl of competent cells were spread onto LBA-Xgal-IPTG plates and 100 µl of a 1/10 dilution of the uncut vector reaction were plated as a positive control. For the rest of the completed ligation reactions, 100 µl of each reaction were spread onto each of four LBA-Xgal-IPTG plates. For the comparable completed ligation controls (cut vector), 100 µl of each reaction were plated.

The LBA-Xgal-IPTG plates were incubated at 37°C for 18 hours. The transformants were scored and the white colonies, which were the potential transformants with the insertion in the MCS region, were restreaked for single colonies onto LBA plates. These plates were prepared by adding 4 ml of sodium ampicillin stock solution (25 mg/ml) to 1 liter of LB agar before pouring the plates. The LBA plates were incubated at 37°C overnight.

6. Minipreps

Miniplasmid isolation and restriction analysis were done to confirm the cloning of the fragments. Single colonies from the LBA plates were cultured overnight in 2 ml of liquid LBA (containing 100 µg/ml of sodium ampicillin) medium (37°C at 125 rpm). The next morning, 1 ml of overnight culture was centrifuged for 30 sec in a 1.5 ml microcentrifuge tube to harvest the cells, the supernatant was discarded, and the pellet was resuspended in 100 µl of a freshly prepared lysis solution (1 M TRIS-HCl pH 8.0, 0.2 M EDTA, 20% glucose, and 0.2% lysozyme). The tube was placed on ice for 5 min, 150 µl of 3 M sodium acetate were added with mixing, and the lysate was left on ice for 45 min. The lysate was centrifuged at maximum speed for 5 min, the supernatant was transferred to a new microcentrifuge tube, extracted once with phenol, once with phenol:chloroform (7:3), and once with chloroform. To precipitate the nucleic acids, 1 ml of 95% ethanol was added and the tube was held at -20°C for 30 min. The precipitate was centrifuged for 20 min and the supernatant was discarded; the pellet was washed once with 70% ethanol and then air dried. The dry pellet was resuspended in 50 µl of TE pH 7.5.

In the restriction analysis, *Eco*RI/*Pst*I double digestion was used for the intact amplified fragment clones. The digestion reaction contained 1 µg of isolated plasmid DNA, 3 µl of 10X Reaction 3 buffer, 10 units of *Eco*RI, and 10 units of *Pst*I; the total volume was 30 µl. For the *Kpn*I fragment clones, *Kpn*I digestion was used; the digestion reaction (total volume of 20 µl) contained 1 µg of isolated plasmid DNA, 2 µl of 10X Reaction 4 buffer, and 10 units of *Kpn*I. For the *Sau*3A fragment clones, *Eco*RI/*Pst*I double digestion was used; the digestion reaction (total volume of 30 µl) contained 1 µg of isolated plasmid DNA, 3 µl of 10X Reaction 3 buffer, 10 units of *Eco*RI, and 10 units of *Pst*I. All of these digestion reactions were incubated at 37°C for 3 hours, then loaded onto 0.8% agarose minigels along with lambda DNA (*c*lind1ts857*S*am7) size markers and electrophoresis was carried out at 50 volts for 4 hours.

7. DNA sequencing of the *hom3-10* gene

a. Preparation of minipreps for sequencing

The minipreps were prepared for DNA sequencing by growing 5 ml overnight cultures of selected insert-bearing plasmid clones in liquid LBA at 37°C in an incubator shaker at 125 rpm, pelleting the cells by centrifuging the cultures at 4000 rpm for 10 min (Damon/IEC Division HN-SII clinical centrifuge), resuspending each pellet of cells in 1.5 ml of STE (100 mM NaCl, 10 mM TRIS-HCl pH 7.5, and 1 mM EDTA), and then transferring the suspensions to 1.5-ml microcentrifuge tubes. The cells in the microcentrifuge tubes were pelleted at maximum speed for 5 min, the pellets were resuspended in 200 µl of TGE (25 mM TRIS-HCl pH 8.0, 1% glucose, and 10 mM EDTA), and the cells were incubated on ice for 5 min. Then 400 µl of freshly prepared alkaline lysis solution (0.2 M NaOH and 1% SDS) were added, the tubes were inverted several times and incubated on ice for another 5 min. After this, 300 µl of cold 3 M potassium acetate pH 5.8 were added, the preparations were mixed gently and incubated on ice for 20 - 30 min.

The suspensions of lysed cells were centrifuged for 5 min in a microcentrifuge and the supernatants were decanted into new microcentrifuge tubes. The supernatants were boiled for 60 sec and then centrifuged for 5 min. These supernatants were then transferred to new microcentrifuge tubes, 750 µl of cold isopropanol were added to precipitate the nucleic acids, the preparations were mixed well and then centrifuged for 10 min. Finally, the supernatants were discarded and the excess droplets were removed from the tubes with tissue. The pellets were redissolved in 200 µl of sterile distilled/deionized water for 40 min by repeated pipetting, and then 200 µl of LiCl/MOPS buffer (5M LiCl and 50mM MOPS) were added to precipitate the RNA. LiCl/MOPS buffer is made by dissolving 21.2 g LiCl and 1.04 g MOPS (4-morpholinepropanesulfonic acid) in 90 ml of water, adjusting the pH to 8.0 with 10 N NaOH, and bringing the final volume to 100 ml with water.

The suspensions were mixed well, incubated on ice for 15 min, centrifuged for 5 min, and the supernatants were transferred to new tubes; 900 µl of 95% ethanol were added and the suspensions were then centrifuged for 10 min. The supernatants were discarded, the walls of the tubes were dried with tissue, the pellets were dissolved in 100 µl of TE pH 7.5, and 1 µl of RNaseA stock solution (10 mg/ml) was added. This mixture was incubated at 37°C for 4 min, then 10 µl of 3 M sodium acetate pH 7.0 were added, and this was followed by two extractions with phenol:chloroform: isoamylalcohol (50:48:2) and one extraction with chloroform. After the addition of 200 µl of 95% ethanol, the nucleic acids were precipitated at -20°C for 20 min. The precipitates were then centrifuged for 15 min, the supernatants were discarded, and the pellets were washed once with 70% ethanol and air dried. The dry pellets were dissolved in 50 µl of TE pH 7.5 and 2 µl were electrophoresed on a 0.8% agarose minigel to be quantified by comparing a sample of plasmid DNA with 0.2 µg of pBR322 DNA. The rest of the sample was stored at -20°C.

b. Preparation of sequencing gels

For sequencing, both 6% and 4% polyacrylamide sequencing gels were used. The 6% (or 4%) acrylamide/urea stock was prepared by dissolving 60 g (or 40 g) of acrylamide and 2 g of bisacrylamide in 100 ml of 10X TBE (108 g TRIS base, 55 g boric acid, and 7.4 g EDTA in 1 liter of water), adding 500 ml of distilled water and 420 g of urea, stirring until everything had dissolved, and then bringing the volume to 1 liter with distilled water. This stock solution was filtered through Whatman No. 3 filter paper and stored at 4°C in a brown bottle.

Spacers were placed between two thoroughly clean and dry glass plates (one long back plate and one short front plate) and when the plates were assembled, they were clamped together. To prepare the gel, 40 µl of 25% ammonium persulfate solution (a catalyst for crosslinking) and 40 µl of TEMED (*N,N,N',N'*-tetramethylethylenediamine, an initiator of crosslinking) were added to 40 ml of 6% (or 4%) acrylamide/urea stock, and this solution was poured between the glass plates. The gels polymerized in 40 - 60 min and were then ready for use.

c. Sequencing reactions

Double-stranded template sequencing was carried out. To an aliquot containing 4 - 5 µg of plasmid DNA, 1/10 volume of 10X denaturant (2 M NaOH and 2 mM EDTA) was added and this mixture was incubated at room temperature for 5 min; then 1/10 volume of 2 M ammonium acetate was added, followed by 2.5 volumes of 95%

ethanol, an incubation at -20°C for 5 min, and pelleting in the microcentrifuge for 10 min. All of the supernatant was removed carefully, the pellet was air dried, and the pellet of dry denatured DNA was resuspended in 8 μl of annealing mix (25 mM TRIS-HCl pH 8.5 and 15 mM $MgCl_2$, plus 15 ng of primer). For the *Kpn*I and *Sau*3A clones, universal primer (5'-GTAAAACGACGGCCAGT-3') and reverse primer (5'-ACAGGAAACAGCTATGA-3') were used, whereas for the intact fragment clones, the primers for PCR (primers A and B) were used. For confirming the mutation site, primer C (5'-CGGTACCATTATCTAC-3') was also used (Fig. 1). By using primers B and C, both strands of DNA in the region where the mutation was found could be sequenced. The primer/template mix was incubated at 37°C for 15 min. Into each of four microcentrifuge tubes (labeled A, C, G, andT) containing 2 μl of sequencing mixture (0.8 units of the Klenow fragment of *Escherichia coli* DNA polymerase I, 2 μCi of ^{35}S dATP (Amersham Corporation), plus 1.5 μl of sequencing mix which contains 13 mM dithiothreitol and 13 mM TRIS-HCl pH 8.5), 2 μl of the primer/template mix were added, followed by 2 μl of each dideoxy NTP/deoxy NTP mixture (40/1 for ddATP/dATP, 7.6/1 for ddCTP/dCTP, 16.8/1 for ddGTP/dGTP, and 32.8/1 for ddTTP/dTTp) to each appropriate tube (*e.g.*, A mix to the tube labeled A, C mix to the tube labeled C, *etc.*). The reaction mixtures were incubated at 42°C for 20 min, then 2 μl of dNTP chase mix (0.5 mM each of dATP, dCTP, dGTP, and dTTP, plus 1 unit of the Klenow fragment of *Escherichia coli* DNA polymerase I) were added, and the reaction mixtures were incubated at 42°C for 15 min; 5 μl of gel loading buffer (0.1 mM EDTA, 0.1% xylene cyanol FF, and 0.1% bromophenol blue in formamide) were added and the mixtures were boiled for 3 min.

d. Acrylamide gel electrophoresis

The gel was clamped, together with a metal heat distribution plate, into a gel stand. The upper and lower reservoirs of the gel stand were filled with buffer (1X TBE) and the stand was then connected to a power supply, with the positive terminal connected to the lower reservoir and the negative terminal connected to the upper reservoir. The gel was prewarmed by running it at 40W of constant power for 30 min; the power was then turned off, the stand was disconnected, and the wells were washed out with 1X TBE buffer using a Pasteur pipette; 4 μl of each sequencing reaction (A, C, G, T) were loaded into each slot, the gel stand was connected to the power supply, and the gel was run at 40W of constant power.

When using a 6% polyacrylamide gel, the gel was run for 1 hour after the blue tracking dye (bromophenol blue) had migrated out of the gel, whereas with a 4% polyacrylamide gel, the gel was run for 2.5 hours after the blue tracking dye had migrated out of the gel. The total time of electrophoresis for a 6% gel was about 3 hours and for a 4% gel it was about 4.5 hours. After disconnecting the gel stand from the power supply, the buffer in the lower reservoir was suctioned into a sidearm flask and disposed of as radioactive waste. After removing the spacers, the glass plates were pried apart carefully by inserting a spatula between them; the plate with the adhering gel was placed, with the gel-side up, on the bench and a sheet of Whatman 3MM chromatography paper was placed onto the gel. The paper was peeled back carefully and the gel adhered to it.

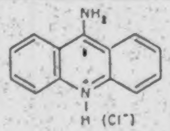
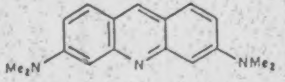
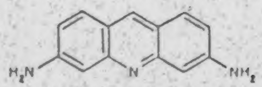
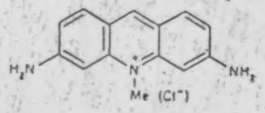
The gel was covered with plastic wrap and put on a gel drier (Bio-Rad Laboratories, Model 583) at 80°C for 2 hours. After the plastic wrap was removed, the gel was put into an X-ray cassette (Kodak X-Omatic) along with a sheet of Fuji X-ray film. The cassette was left at room temperature for 2 - 3 days and the X-ray film was then developed (3 min in Kodak developer, 3 sec in 3 - 5% acetic acid stop solution, and then 2 min in Kodak fixer), rinsed in distilled water, and air dried. The DNA sequence was then read from the autoradiograph.

Table 1 The limiting media used for the compartmentalization tests

Allele	Limiting amino acid	Standard concentration (mg/L)	Limiting concentration[a] (mg/L)
arg4-17	L-arginine L-adenine	20 20	1.0 5.0
his1-7	L-histidine	20	0.2
his4-38	L-histidine	20	3.2
hom3-10	L-methionine	20	0.5
leu2-3	L-leucine	20	5.0

[a] Previously established limiting concentrations were used for *leu2-3* (P.O. Andersson, E.A. Savage, and U.G.G. Hennig, unpublished data), *arg4-17*, and *his1-7* (von Borstel *et al.*, 1971).

Table 2 Acridines and acridine salts used in the compartmentalization tests

Acridine	CAS Registry No.	Chemical structure	Acridine salt	CAS Registry No.
9-Aminoacridine (MW 194.23)	90-45-9		9-Aminoacridine hydrochloride (MW 230.68)	578-07-4
Acridine orange (MW 265.36)	494-38-2		-	-
Acriflavine[a] (a mixture of two acridines)	8048-52-0		Proflavine (MW 209.25)	92-62-6
			Proflavine methochloride (MW 259.74)	Not registerd

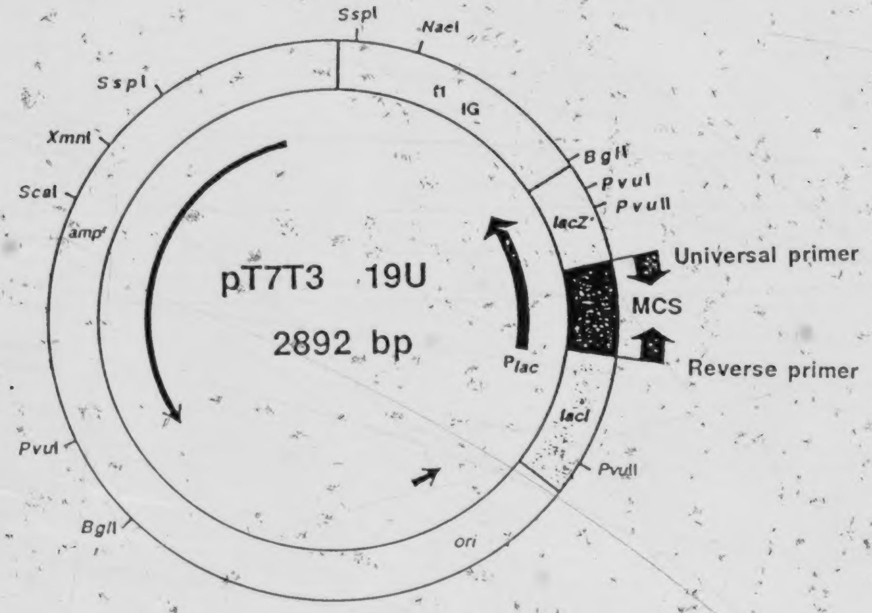
---

[a] Acriflavine neutral is a mixture of proflavine (3,6-diaminoacridine) and proflavine methochloride (3,6-diamino-10-methylacridinium chloride). A MW of 226.2 is commonly used for acriflavine (Arakatsu, 1971; Heslot *et al.*, 1970; Mahler, 1973; McClarg and Bowers, 1968).

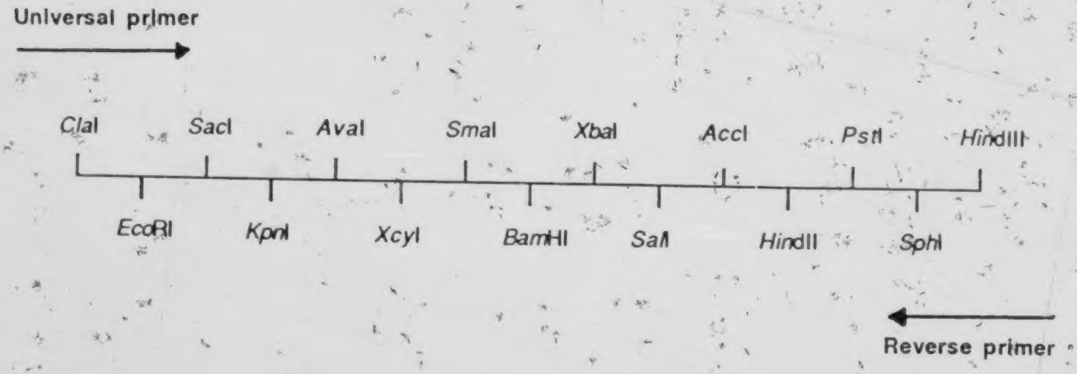
Fig. 1. The wild-type sequence of the *HOM3* gene (from Rafalski and Falco, 1988). Primers A and B, which were used for PCR and DNA sequencing, are indicated by the rectangles (▭). The size of the region spanned by primers A and B is 1.3 kb, of which 1.2 kb is the coding region. Primer C, which was used in DNA sequencing to confirm the mutation, is shown in square brackets ([ ]). The *Kpn*I restriction sites are indicated with double lines (═); the *Kpn*I fragment is 0.76 kb. The *Sau*3A restriction sites are indicated with single lines (───); the *Sau*3A fragment is 0.36 kb.

```
    1  GGATCCTTTTCTTTCTAGAACTCCCCTAATATCAACTTTTTACTCTACTGTGGCTCTAACCACGTGTATTTTTCAGGCCCATGACTCACC  90
   91  TTTTCTTTCTTTTTCACTAGCAAACGAAAATTTCATCATCACTTTTTTTTTTTTTCACTTTCCGTACGCAGTCTTCTCCAGAGATTCTTT  180
  181  TAGCTTCGAAGAAATAATGTATAATATCCATATATAAGAATACTGCTGGATAATTATTTTATTTCTATTTTAACAGTGCTCTACTCATTG  270
                               A
  271  AAGGATATTTGTGTACCCAATACTCTCTCCATCGCTTAAGCTCACATAGCTATCGCATTTTATCACACAAGCTTTCATTTTTTTTAACTT  360
  361  TTACATGCCAATGGATTTCCAACCTACATCAAGTCATTCGAACTGGGTCGTGCAAAAGTTCGGTGGTACATCTGTCGGTAAATTTCCCGT  450
             M  P  M  D  F  Q  P  T  S  S  H  S  N  W  V  V  Q  K  F  G  G  T  S  V  G  K  F  P  V
  451  CCAAATAGTGGATGACATTGTGAAGCACTATTCTAAACCTGACGGCCCAAACAATAATGTCGCTGTCGTTTGTTCCGCCCGTTCTTCATA  540
        Q  I  V  D  D  I  V  K  H  Y  S  K  P  D  G  P  N  N  N  V  A  V  V  C  S  A  R  S  S  Y
  541  CACCAAGGCTGAAGGTACCACTTCTCGTCTTTTGAAATGTTGTGATTTGGCTTCGCAAGAATCTGAATTTCAAGACATTATCGAAGTTAT  630
        T  K  A  E  G  T  T  S  R  L  L  K  C  C  D  L  A  S  Q  E  S  E  F  Q  D  I  I  E  V  I
  631  CAGACAAGACCATATCGATAATGCCGACCGGTTCATTCTCAATCCTGCCTTGCAAGCCAAGTTAGTGGATGATACCAATAAAGAACTTGA  720
        R  Q  D  H  I  D  N  A  D  R  F  I  L  N  P  A  L  Q  A  K  L  V  D  D  T  N  K  E  L  E
  721  ACTGGTCAAGAAATATTTAAATGCTTCAAAAGTTTTGCGTCAAGTGAGTTCACGTACAGTAGATCTGGTGATGTCATGTGGTGAGAAGTT  810
        L  V  K  K  Y  L  N  A  S  K  V  L  G  E  V  S  S  R  T  V  D  L  V  M  S  C  G  E  K  L
  811  GAGTTGTTTGTTCATGACTGCTTTATGTAATGACCGTGGCTGTAAGGCCAAATATGTGGATTTGAGCCACATTGTTCCCTCTGATTTCAG  900
        S  C  L  F  M  T  A  L  C  N  D  R  G  C  K  A  K  Y  V  D  L  S  H  I  V  P  S  D  F  S
  901  TGCCAGCGCTTTGGATAACAGTTTCTACACTTTCCTGGTTCAAGCATTGAAAGAAAAATTGGCCCCCTTTGTAAGTGCTAAAGAGCGTAT  990
        A  S  A  L  D  N  S  F  Y  T  F  L  V  Q  A  L  K  E  K  L  A  P  F  V  S  A  K  E  R  I
  991  CGTTCCAGTCTTTACAGGGTTTTTTGGTTTAGTTCCAACTGGTCTTCTGAATGGTGTTGGTCGTGGCTATACCGATTTATGTGCCGCTTT  1080
        V  P  V  F  T  G  T  F  G  L  V  P  T  G  L  L  N  G  V  G  R  G  Y  T  D  L  C  A  A  L
 1081  GATAGCAGTTGCTGTAAATGCTGATGAACTACAAGTTTGGAAGGAAGTTGATGGTATATTTACTGCTGATCCTCGTAAGGTTCCTCAAGC  1170
        I  A  V  A  V  N  A  D  E  L  Q  V  W  K  E  V  D  G  I  F  T  A  D  P  R  K  V  P  E  A
 1171  ACGTTTGCTAGACAGTGTTACTCCAGAAGAAGCTTCTGAATTAACATATTATGGTTCCGAAGTTATACATCCTTTTACGATGGAACAAGT  1260
        R  L  L  D  S  V  T  P  E  E  A  S  E  L  T  Y  Y  G  S  E  V  I  H  P  F  T  M  E  Q  V
                                                                                C
 1261  TATTAGGGCTAAGATTCCTATTAGAATCAAGAATGTTCAAAATCCATTAGGTAACGGTACCATTATCTACCCAGATAATGTAGCAAAGAA  1350
        I  R  A  K  I  P  I  R  I  K  N  V  Q  N  P  L  G  N  G  T  I  I  Y  P  D  N  V  A  K  K
 1351  GGGTGAATCTACTCCACCACATCCTCCTGAGAACTTATCCTCATCTTTCTATGAAAAGAGAAAGAGAGGTGCCACTGCTATCACCACCAA  1440
        G  E  S  T  P  P  H  P  P  E  N  L  S  S  S  F  Y  E  K  R  K  R  G  A  T  A  I  T  T  K
 1441  AAATGACATTTTCGTCATCAACATTCATTCCAATAAGAAAACCCTATCCCATGGTTTCCTAGCTCAAATATTTACCATCCTGGATAAGTA  1530
        N  D  I  F  V  I  N  I  H  S  N  K  K  T  L  S  H  G  F  L  A  Q  I  F  T  I  L  D  K  Y
 1531  CAAGTTAGTCGTAGATTTAATATCTACTTCTGAAGTTCATGTTTCGATGGCTTTGCCATTCCAGATGCAGACTCATTAAAATCTCTGAGA  1620
        K  L  V  V  D  L  I  S  T  S  E  V  H  V  S  M  A  L  P  F  Q  M  Q  T  H  •
          B
 1621  CAAGCTGAGGAAAAATTGAGAATTTTAGGTTCTGTTGATATCACAAAGAAGTTGTCTATTGTTTCATTAGTTGGTAAACATATGAAACAA  1710
 1711  TACATCGGCATTGCTGGTACCATGTTTACTACTCTTGCTGAAGAAGGCATCAACATTGAAATGATTTCTCAAGGGGCAAATGAAATAAAC  1800
 1801  ATATCCTGCGTTATCAATGAATCTGACTCCATAAAAGCCGCTACAATGTATTCATGCCAAGTTACTAAGTGAGCGGACAAATACTTCAAAC  1890
 1891  CAATTTGAACATGCCATTGATGAACGTTTAGAACAATTGAAAAGACTTGGAATTTAAATCCACCTTTCTTCTTCACTTTAATGATAGAAT  1980
 1981  ATTAATTTTCCCTTTATATATATATATATATATATACGCGCACATATATTTACATATATATTAATGTTTTGTAACCTATATTGCGGCCG  2070
 2071  ATTATTCCCTAAATGTAATGTCAGGGTTATACTGTATCAATTGCTAAAGTAGATCTCATTCATCGAGAGCATAGGATGAAATAAAAGATA  2160
 2161  CAAGAACGTGGCGGACAAGAAGAAATTTTACGAAATAAGATCGGCTAAAGCTTTCGCTAAATGCTTCTTTGAAGTGAAATTTAACGGCTT  2250
 2251  GTACTTAACTTCAGGTTTTATAACGTATGCATGAAGTGATTGAATTGCGCAATTAGTGGTCCTGGAAAGCAAATCCCGATCACTGACATC  2340
 2341  ACTAGTGATTTTATTTTTATCAAGCGCAAGATACTCGCATAATTTACCCATCACTTGATCAAACTTCGGGAAGGTAAATCTTCTAGGAAC  2430
 2431  CTTTGTTAACTTTTCAATTTCCACAATTTTAAGGTGTCTCTCCAAATCACGGATGGTTAAAAAGTTGCCTCCAAATACCAAAGAGTCTAC  2520
 2521  TGGTGTATAAACTGCATGAATATATCCAGCTG  2552
```

Fig. 2. The pT7T3 19U cloning vector. The entire PCR amplified 1.3 kb fragment of the *hom3-10* gene was cloned into the *Sma*I site, the 0.76 kb *Kpn*I fragment was cloned into the *Kpn*I site, and the 0.36 kb *Sau*3A fragment was cloned into the *Bam*HI site. Universal primer and reverse primer were used in DNA sequencing.


SspI
NaeI
SspI
XmnI
ScaI
amp^r
f1
IG
BglI
PvuI
PvuII
lacZ'
Universal primer
MCS
Reverse primer
pT7T3 19U
2892 bp
P_lac
lacI
PvuII
PvuI
BglI
ori

Multi-cloning site (MCS) :


Universal primer
ClaI
SacI
AvaI
SmaI
XbaI
AccI
PstI
HindIII
EcoRI
KpnI
XcyI
BamHI
SalI
HindII
SphI
Reverse primer

Universal primer: 5'-GTAAAACGACGGCCAGT-3'

Reverse primer : 5'-ACAGGAAACAGCTATGA-3'

RESULTS

A. The limiting amino acid for the *hom3-10* locus

Since the mutation in the *HOM3* gene results in a requirement for both threonine and methionine for growth, each of these two amino acids was tested as a limiting amino acid for measuring the reversion rate of *hom3-10* by the compartmentalization test. The data in Table 3 show that threonine is not suitable for use as a limiting amino acid. At the lower concentrations (1.0 mg/L), although there is no continuous reversion, the number of compartments with revertant colonies is zero, therefore the reversion rate cannot be calculated. At higher concentrations (1.5, 2.0, 2.5, and 3.0 mg/L), because of the continuous reversion, the reversion rate cannot be calculated.

Methionine is optimum as a limiting amino acid at a concentration of 0.5 mg/L (Table 3). At this concentration, there is no continuous reversion and the number of compartments with revertant colonies is optimum as well, therefore the reversion rate can be calculated accurately. The other limiting concentrations of methionine cannot be used because at lower concentrations (0.1 and 0.3 mg/L) the number of compartments with revertant colonies is zero, and at higher concentrations (0.7, 0.9, and 1.1 mg/L) there is continuous reversion.

B. Reversion studies with 9-aminoacridine, acriflavine, and acridine orange

1. Effects on cell generation time and viability

As shown in Table 4 and Fig. 3, there is a concentration range of 9-aminoacridine (5 µg/ml - 100 µg/ml) in which the cell generation time was delayed without a concurrent decrease in viability. In the control experiments (cells that were not treated with 9-aminoacridine), the cell generation time is 1.83 hours; with increasing concentrations of 9-aminoacridine, the generation time increased. The results of the viability experiments show that 9-aminoacridine, at concentrations between 5 μg/ml and 100 μg/ml, did not decrease the cell viability. In this concentration range, the cell generation time increased from 1.82 hours to 3.53 hours while the cell viability remained the same (about 92%). When the concentration was higher than 100 μg/ml, the delay in cell generation time was accompanied by a decrease in cell viability.

In contrast to 9-aminoacridine, acriflavine and acridine orange do not show concentration ranges in which the cell generation time was delayed without a concurrent decrease in viability. For acriflavine, at concentrations ranging from 1.0 μg/ml to 12 μg/ml, the cell generation time increased from 1.9 hours to 4.2 hours and was accompanied by a decrease in viability from 96% to 29.1% (Table 5). As shown in Fig. 4, once the cell generation time was delayed (1.0 μg/ml), the cell viability started to decrease as well. For acridine orange, at concentrations ranging from 2.5 μg/ml to 10.0 μg/ml, the cell generation time increased from 1.90 hours to 4.25 hours with a concurrent decrease in viability from 93% to 29% (Table 5, Fig. 5).

2. Effect on mutation rate

The mutation rate is defined as mutational events/cell/generation. 9-Aminoacridine does not affect the mutation rate of the two base substitution alleles, *his1-7* and *arg4-17*. The spontaneous reversion rate for *his1-7* is 6.8×10^{-8}. Using various concentrations of 9-aminoacridine, this reversion rate for *his1-7* was not altered (Table 6). The spontaneous locus reversion rate for *arg4-17* is 0.14×10^{-8}, and the spontaneous suppressor reversion rate is 1.62×10^{-8}. These two reversion rates were not affected by 9-aminoacridine (Table 6).

9-Aminoacridine does affect the reversion rate for *hom3-10* and this effect is dose dependent. As shown in Table 7 and Fig. 6, at lower concentrations (10 µg/ml - 30 µg/ml), 9-aminoacridine is mutagenic; it increased the mutation rate 6-fold (from the spontaneous reversion rate of 0.8×10^{-8} to 4.32×10^{-8}) at 32.5 µg/ml. At higher concentrations (35 µg/ml - 80 µg/ml), 9-aminoacridine is antimutagenic; it decreased the mutation rate by more than 2-fold (from the spontaneous reversion rate of 0.8×10^{-8} to 0.36×10^{-8}) at 80 µg/ml. The mutagenic and antimutagenic effects were also detected with the reversion of the other two frameshift alleles, *his4-38* and *leu2-3* (Table 7).

Just as with 9-aminoacridine, acriflavine and acridine orange do not affect the reversion rates of the two base substitution alleles, *his1-7* and *arg4-17*, but are mutagenic when *hom3-10* is analyzed (Table 8). At concentrations of 1.0 µg/ml, 2.0 µg/ml, and 8.0 µg/ml of acriflavine, the reversion rate for *hom3-10* increased up to 4.5-fold. At concentrations of 1.0 µg/ml, 2.5 µg/ml, and 5.0 µg/ml of acridine orange, the reversion rate for *hom3-10* increased 3-fold. In contrast to 9-aminoacridine, acriflavine and acridine orange do not show an antimutagenic effect at any of the concentrations that were tested.

C. Molecular studies on the *hom3-10* allele

1. PCR and cloning

Using the genomic DNA of strains XV185-14C and XV195-6B as a template, the fragment containing the *hom3-10* gene was amplified by PCR and the result is shown in Fig. 7. By comparison to the lambda DNA (*c*Ind1ts857Sam7) size markers in lane 1, the amplified fragment is 1.3 kb long. The size indicates that the amplified fragment is the portion of the *hom3-10* gene which is spanned by primers A and B, and of which 1.2 kb is the coding region of *hom3-10* (Fig. 1).

Following the extraction and purification of the amplified DNA fragments from 0.9% low melting agarose gels, cloning was carried out. As illustrated in Fig. 1 and Fig. 2, the amplified 1.3 kb intact fragment was expected to be cloned into the *Sma*I site of the MCS region of the pT7T3 19U vector, the 0.76 kb *Kpn*I fragment into the *Kpn*I site of the MCS, and the 0.36 kb *Sau*3A fragment into the *Bam*HI site of the MCS.

For the transformation experiments, the transformation efficiency with strain JM83 of *Escherichia coli* was between 2.5×10^5 and 1.0×10^6 transformants/μg of intact pT7T3 DNA. In the cloning of the blunt-ended DNA fragment (the intact PCR amplified 1.3 kb fragment), although the ligation reaction was "driven" by a high concentration of fragment DNA and a high concentration of T4 ligase, the cloning efficiency was still relatively low when compared with the cloning efficiency of the cohesive-ended DNA fragments (the 0.76 kb *Kpn*I fragment and the 0.36 kb *Sau*3A fragment). The potential transformants with expected insertions were selected (white colonies on LBA-Xgal-IPTG plates) in each transformation experiment and analyzed by restriction analysis.

As shown in Fig. 8, each 1.3 kb fragment clone was confirmed by double digestion of the potential transformant with *Eco*RI and *Pst*I. The recognition sequence for *Sma*I is 5'-CCC⬇GGG-3' but after the *Sma*I digested vector had been ligated with the 1.3 kb fragment, this recognition site was abolished. Since the *Sma*I site is flanked by *Eco*RI and *Pst*I sites in the MCS region, and the insertion at the *Sma*I site does not affect the *Eco*RI and *Pst*I sites, double digestion of the transformant with the insertion at the *Sma*I site should release two fragments, which are the insertion and the vector fragment. As shown in Fig. 8 (lane 2), the insertion is 1.3 kb long which indicates that the intact amplified fragment has been cloned into the *Sma*I site.

The 0.76 kb *Kpn*I fragment clones were confirmed by *Kpn*I digestion. The recognition sequence for *Kpn*I is 5'-GGTAC⬇C-3'; after the ligation of the *Kpn*I digested vector with the 0.76 kb *Kpn*I fragment, two *Kpn*I sites flank the insertion. Therefore *Kpn*I digestion of the transformant with the insertion at the *Kpn*I site should release two fragments, which are the insertion and the vector fragment. As shown in Fig. 8 (lane 3), the insertion is 0.76 kb which indicates that the insertion is the *Kpn*I fragment.

The 0.36 kb *Sau*3A fragment clones also were confirmed by *Eco*RI/*Pst*I double digestion. This *Sau*3A fragment was expected to be cloned into the *Bam*HI site of the vector. *Bam*HI recognizes the 6-base sequence 5'-G⬇GATCC-3' whereas *Sau*3A recognizes the 4-base sequence 5'-⬇GATC-3'. After the ligation of the *Bam*HI digested vector with the *Sau*3A fragment, the *Bam*HI recognition sites were abolished. *Sau*3A digestion was not used to confirm the clone because there are more than 10 *Sau*3A sites in the vector. In the MCS region, the *Bam*HI site is also flanked by *Eco*RI and *Pst*I sites, therefore *Eco*RI/*Pst*I double digestion was carried out to confirm the *Sau*3A fragment clones. As seen in Fig. 8 (lane 4), two fragments were released, and these are the 0.36 kb insertion and the 2.9 kb vector.

2. Sequence analysis

a. Frameshift mutation

The base change was deduced by comparing the *hom3-10* sequence with the published wild-type sequence (Rafalski and Falco, 1988). Both strands of the region in which the mutation was found were sequenced by using primers B and C (Fig. 1). The mutation in *hom3-10* is the insertion of one C in a proline codon. This position is 18 bp from the normal stop codon.

b. Misincorporation of bases by *Taq* DNA polymerase during PCR

It has been reported that *Taq* DNA polymerase has a lower fidelity in DNA synthesis when compared with the Klenow fragment of *Escherichia coli* DNA polymerase I and the overall error frequency of misincorporation is 0.25%, which is about 3 times higher than that with the Klenow fragment (Saiki *et al.*, 1988). Out of a total of 3 kb of DNA sequences read, 8 misincorporations were detected (Table 9), and this translates into an error frequency of 0.27%.

Amplified DNA fragments from 3 or 4 different PCR experiments were cloned and sequenced. By comparing the sequences of the same fragment from several different clones, the misincorporation of bases can be discerned. In Table 9, all the mutations that were introduced by *Taq* DNA polymerase, as well as the actual *hom3-10* mutation, are listed. By using primers A and B (for sequencing the intact amplified clones), and universal primer and reverse primer (for sequencing the *Kpn*I and *Sau*3A clones), 200 bases could be read after sequencing with each primer. An insertion of a C was found in each of the four amplified 1.3 kb intact fragment clones. This consistency indicates that this is the actual *hom3-10* mutation.

Double-stranded DNA sequencing was carried out for two of the four clones, and it was found that there is a G insertion at the site corresponding to the C insertion on the opposite strand. The misincorporations introduced by the *Taq* DNA polymerase are point mutations, and both transitions and transversions were observed. There does not seem to be a sequence preference for the misincorporation to take place. In 10 separate clones (4 with the 1.3 kb fragment of amplified DNA, 3 with the 0.76 kb *Kpn*I fragment of amplified DNA, and 3 with the 0.36 kb *Sau*3A fragment of amplified DNA), 8 misincorporated bases were identified. These misincorporations occurred throughout the amplified product and no deletions or insertions, other than the actual frameshift mutation in *hom3-10*, were detected (Table 9).

Table 3 The concentrations of limiting amino acids tested for the *hom3-10* allele, as determined in strain XV185-14C

Limiting amino acid	Concentration (mg/L)	Continuous reversion[a]	Cell number[b] (x10^4 cells/ml)	N_r[c]
Methionine	0.1	-	<1	0
	0.3	-	92	0
	0.5	-	1202	88
	0.7	-	1808	500
	0.9	+	2106	500
	1.1	+	2243	500
Threonine	1.0	-	62	0
	1.5	+	760	500
	2.0	+	930	500
	2.5	+	1040	500
	3.0	+	1184	500

---

[a] Continuous reversion means that after 12 days of incubation, new revertant colonies continue to appear each day.

[b] This cell number was counted on the 12th day.

[c] N_r is the number of compartments with revertant colonies out of the 500 compartments use in each experiment.

Table 4 Cell generation time and viability with increasing concentrations of 9-aminoacridine, as determined in strain XV195-6B

Concentration of 9-aminoacridine		Generation time (hour)				Viability (%)			
µg/ml	µM	# of Expts.	$\bar{x}$	SD	SE$\bar{x}$	# of Expts.	$\bar{x}$	SD	SE$\bar{x}$
0	0	18	1.83	0.10	0.023	18	93.8	6.1	1.4
5	25.6	3	1.82	0.15	0.087	3	93.6	4.2	2.4
10	51.2	5	2.16	0.18	0.081	5	90.8	5.3	2.4
20	102.5	3	2.27	0.19	0.110	3	92.4	6.1	3.5
30	153.7	3	2.47	0.21	0.121	3	93.1	4.0	2.3
35	179.3	3	2.58	0.19	0.110	3	92.6	3.8	2.2
40	204.9	3	2.76	0.22	0.127	3	90.4	3.2	1.9
60	307.4	4	2.83	0.19	0.095	4	93.0	4.6	2.3
80	409.8	3	3.07	0.26	0.150	3	91.5	6.0	3.5
100	512.3	4	3.53	0.22	0.110	4	92.9	2.1	1.1
120	614.8	5	4.03	0.25	0.559	5	70.4	3.3	1.5

Table 5 Cell generation time and viability with increasing concentrations of acriflavine and acridine orange, as determined in strain XV195-6B

Acridine	Concentration		Generation time (hour)				Viability (%)			
	μg/ml	μM	# of Expts.	$\bar{x}$	SD	SE$\bar{x}$	# of Expts.	$\bar{x}$	SD	SE$\bar{x}$
Acriflavine[a]	0.0	0.00	12	1.90	0.05	0.043	12	96.6	2.8	0.8
	0.5	2.26	3	1.85	0.12	0.069	3	92.0	6.8	3.9
	1.0	4.52	4	1.87	0.08	0.090	4	94.7	5.1	2.6
	2.0	9.04	4	2.23	0.17	0.085	4	72.0	4.1	2.1
	4.0	18.08	3	2.40	0.09	0.110	3	61.7	5.6	3.2
	8.0	36.16	5	3.80	0.15	0.067	5	43.0	3.9	1.7
	12.0	53.05	4	4.20	0.26	0.130	4	29.1	1.1	0.6
Acridine orange	0.0	0.00	11	1.85	0.12	0.036	11	92.1	7.1	2.1
	0.2	0.74	3	1.95	0.10	0.058	3	91.8	6.3	3.6
	1.0	3.68	3	1.90	0.09	0.052	3	93.2	6.1	3.5
	2.5	9.20	4	2.77	0.15	0.075	4	62.7	3.2	1.6
	5.0	18.39	3	3.60	0.25	0.144	3	42.5	3.3	1.9
	7.5	27.59	3	4.05	0.30	0.173	3	30.8	2.1	1.2
	10.0	36.78	3	4.85	0.23	0.133	3	23.1	1.1	0.6

---

[a] A MW of 226.2 is commonly used for acriflavine (for references, see the footnote for Table 2).

Table 6 Reversion rates (mutational events/cell/generation) for two base-substitution alleles with increasing concentrations of 9-aminoacridine, as determined in strain XV185-14C

Concentration		*his1-7 -> HIS*+[a]		*arg4-17 -> ARG*+[b]		
μg/ml	μM	# of Expts.	Rate ($x10^{-8}$)	# of Expts.	Locus ($x10^{-8}$)	Suppressor ($x10^{-8}$)
0	0	9	6.84	6	0.14	1.62
5	25.6	3	6.72	3	0.13	1.42
20	102.5	3	6.90	3	0.16	1.56
40	204.9	4	6.46	3	0.14	1.62
60	307.4	3	6.72	2	0.15	1.49
80	409.8	2	6.61	3	0.16	1.26
120	614.8	3	6.43	2	0.12	1.42

[a] For 7 replicates, the mean spontaneous reversion rate is $6.8443x10^{-8}$, the standard deviation (SD) is 0.2968, and the coefficient of variation (CV) is 0.468.

[b] For 4 replicates, the mean spontaneous locus reversion rate is $0.1625x10^{-8}$, the SD is 0.015, and the CV is 0.0923; the mean spontaneous suppressor reversion rate is $1.6255x10^{-8}$, the SD is 0.1497, and the CV is 0.0958.

Table 7 Reversion rates (mutational events/cell/generation) for several frameshift alleles with increasing concentrations of 9-aminoacridine, as determined in strain XV195-6B

Concentration		*hom3-10* -> *HOM*$^{+}$[a]		*his4-38* -> *HIS*$^{+}$		*leu2-3* -> *LEU*$^{+}$	
µg/ml	µM	# of Expts.	Rate($\times10^{-8}$)	# of Expts.	Rate($\times10^{-8}$)	# of Expts.	Rate($\times10^{-8}$)
0.0	0.0	6	0.89	1	0.030	2	0.41
5.0	25.6	3	0.94				
10.0	51.2	3	1.02	1	0.059	1	1.02
20.0	102.5	3	3.32				
30.0	153.7	3	4.20				
32.5	166.5	3	4.32				
35.0	179.3	4	0.42				
40.0	204.9	2	0.37	1	0.017	1	0.13
60.0	307.4	3	0.41				
80.0	409.8	3	0.36				

---

[a] For 9 replicates, the mean spontaneous reversion rate is 0.8922×10^{-8}, the standard deviation (SD) is 0.0319, and the coefficient of variation (CV) is 0.0358.

Table 8 Reversion rates[a] (mutational events/cell/generation) with increasing concentrations of acriflavine and acridine orange

Acridine	Concentration		*arg4-17->ARG*$^{+}$			*his1-7->HIS*$^{+}$		*hom3-10->HOM*$^{+}$	
	µg/ml	µM	# of Expts.	Locus ($\times 10^{-8}$)	Sup. ($\times 10^{-8}$)	# of Expts.	Rate ($\times 10^{-8}$)	# of Expts.	Rate ($\times 10^{-8}$)
	0	0	6	0.15	1.60	5	6.79	4	0.79
Acriflavine[b]	1.0	4.42	3	0.14	1.56	3	6.60	3	0.90
	2.0	8.84	3	0.17	1.63	3	6.52	2	2.62
	8.0	35.37	3	0.16	1.69	3	6.68	3	3.60
	0	0	5	0.16	1.74	6	6.82	3	0.79
Acridine orange	1.0	3.68	3	0.15	1.68	3	6.74	3	0.96
	2.5	9.20	2	0.18	1.66	3	6.60	3	2.34
	5.0	18.39	3	0.16	1.76	3	6.80	2	2.46

---

[a] Reversion rates for the base substitution alleles were measured in strain XV185-14C and reversion rates for *hom3-10* were measured in strain XV195-6B. The statistical evaluation for the spontaneous reversion rates of these alleles are listed in the footnotes for Tables 6 and 7.

[b] Acriflavine neutral is a mixture of proflavine and proflavine methochloride. A MW of 226.2 is commonly used for acriflavine (for references, see the footnote for Table 2).

Table 9 Misincorporation of bases by *Taq*I DNA polymerase during PCR

Cloned fragment[a]	Clone No.	Base change[b]	Position	Flanking sequence	
				5'	3'
1.3 kb intact fragment	1	G -> A	341	GAA	TCC
		C insertion*	1585-1588		
	2	C -> T	466	TGA	ATT
		C insertion*	1585-1588		
	3	T -> C	1485	CCC	ATC
		C insertion*	1585-1588		
	4	**C insertion***	1585-1588		
*Kpn*I	5	A -> C	639	AAG	CCA
	6	None			
	7	G -> A	1266	TTC	GGC
*Sau*3A	8	G -> A	837	TAT	TAA
	9	C -> A	925	TTT	CAC
	10	T -> C	1093	TGC	GTA

[a] The PCR amplified 1.3 kb intact fragment had been cloned into the *Sma*I site of the pT7T3 19U vector, the 0.76 kb *Kpn*I fragment into the *Kpn*I site, and the 0.36 kb *Sau*3A fragment into the *Bam*HI site.

[b] Of the 8 base misincorporations detected 6 were transitions and 2 were transversions. The actual mutation in *hom3-10* is indicated with an asterisk (*).

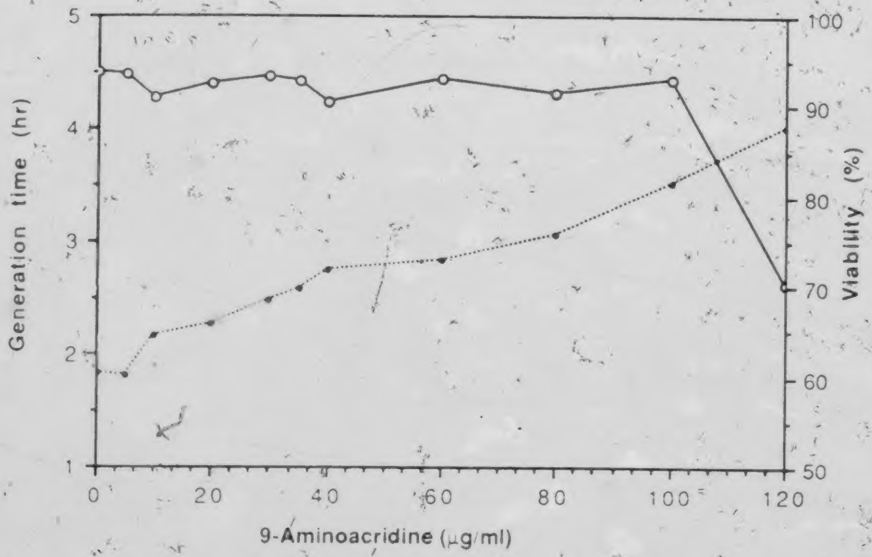


Fig. 3. Cell generation time (---•---) and viability (—o—) in strain XV195-6B with increasing concentrations of 9-aminoacridine. The mean ($\bar{x}$), standard deviation (SD), and standard error of the mean (SE$\bar{x}$) for each point are shown in Table 4. The concentration range in which the cell generation time is delayed without a concurrent decrease in viability is between 5 µg/ml and 100 µg/ml.

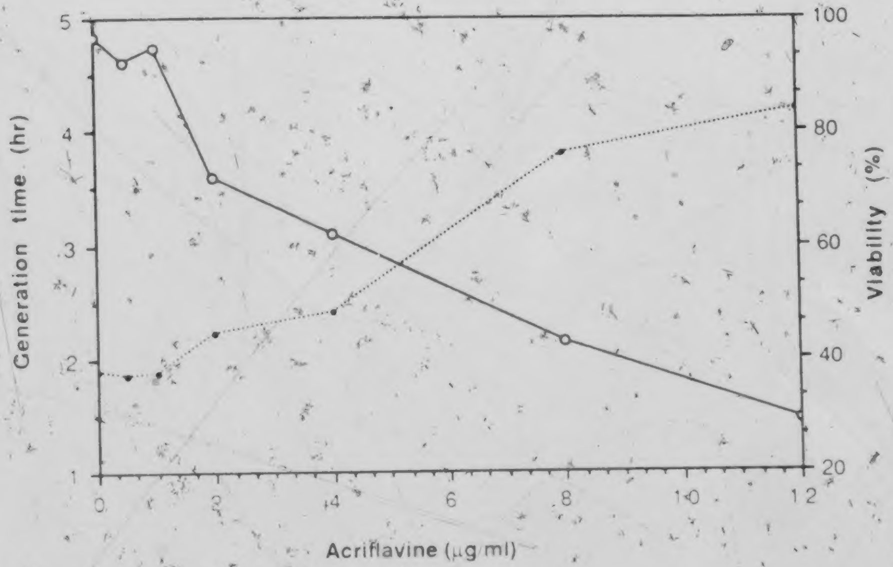


Fig. 4. Cell generation time (-----•-----) and viability (—o—) in strain XV195-6B with increasing concentrations of acriflavine. The mean ($\bar{x}$), standard deviation (SD), and standard error of the mean (SE$\bar{x}$) for each point are shown in Table 5. The delay in cell generation time is accompanied by a decrease in cell viability.

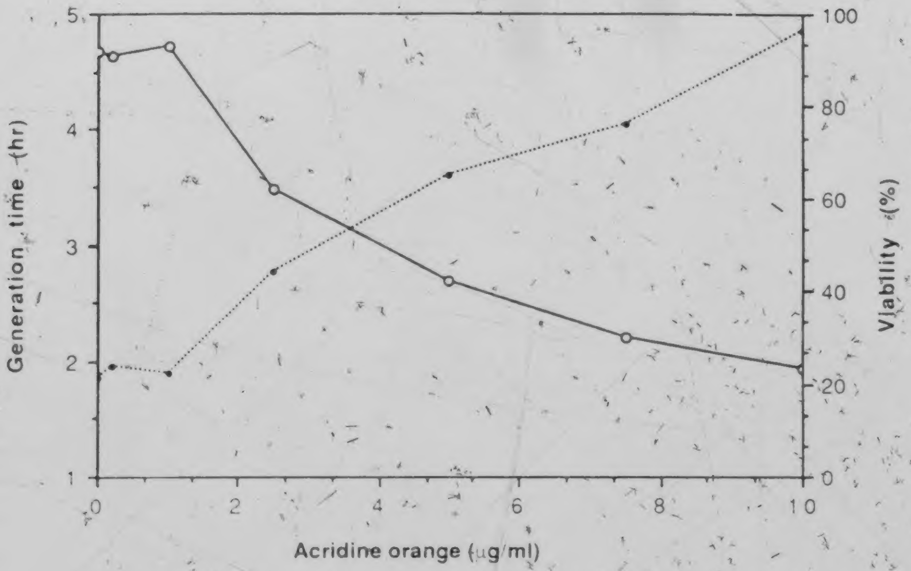


Fig. 5. Cell generation time (---•---) and viability (—o—) in strain XV195-6B with increasing concentrations of acridine orange. The mean ($\bar{x}$), standard deviation (SD), and standard error of the mean ($SE\bar{x}$) for each point are shown in Table 5. There is no concentration at which the cell generation time is delayed without a concurrent decrease in cell viability.

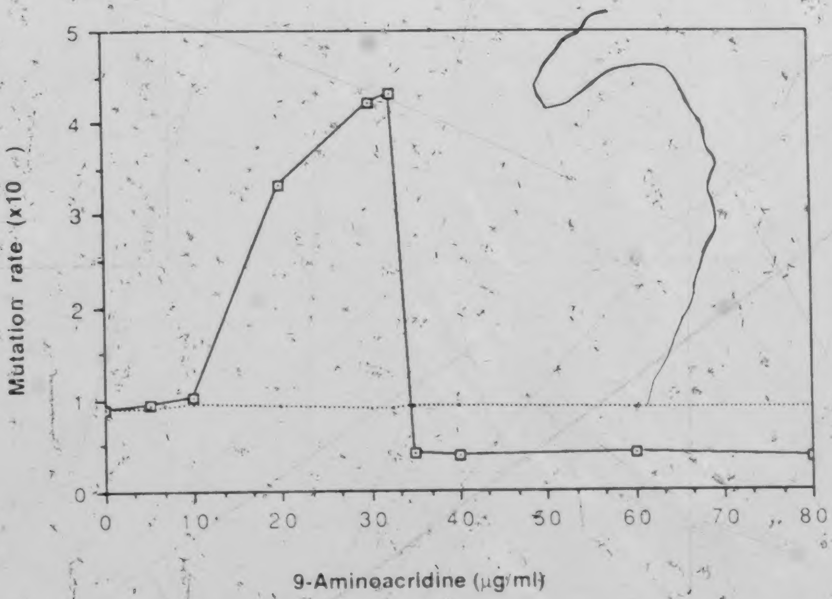


Fig. 6. Reversion rates (mutational events/cell/generation) for *hom3-10* in strain XV195-6B with increasing concentrations of 9-aminoacridine (—▫—). 9-Aminoacridine shows both mutagenic and antimutagenic effects on *hom3-10,* and these effects are dose dependent. At lower concentrations (10 µg/ml - 32.5 µg/ml), 9-aminoacridine is mutagenic; at higher concentrations (35 µg/ml - 80 µg/ml), 9-aminoacridine is antimutagenic. The spontaneous mutation rate for *hom3-10* in strain XV195-6B is 0.89×10^{-8} mutants/cell/generation (········).

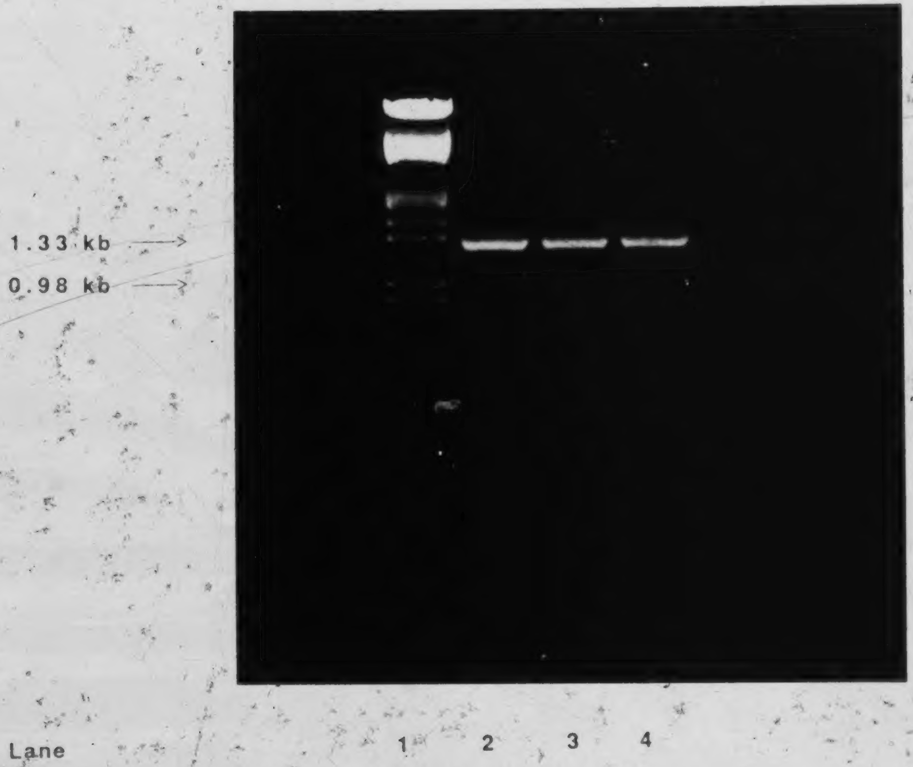


Fig. 7. The *hom3-10* gene, amplified by PCR. Lane 1 contains lambda DNA (cIind1ts857*Sam*7) size markers (*Eco*RI/*Hin*dIII restriction fragments). Genomic DNA from strains XV185-14C (lane 2) and XV195-6B (lanes 3 and 4) was used as a template in PCR. The size of each amplified fragment is 1.3 kb and this indicates that the amplified DNA is the fragment of the *hom3-10* gene which is spanned by primers A and B (see Fig. 1).

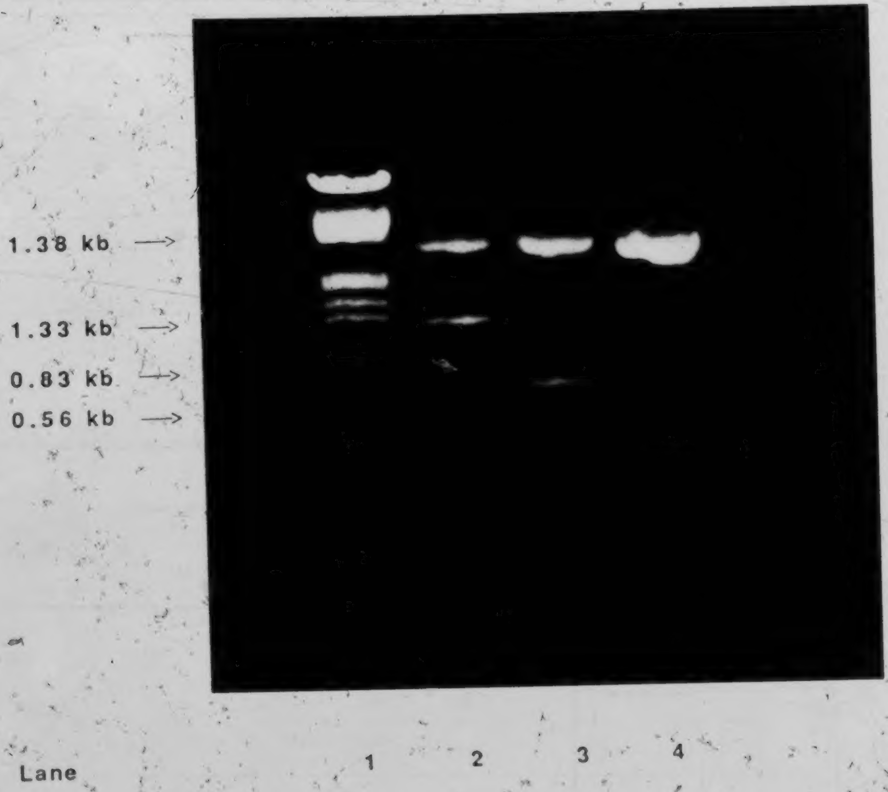


Fig. 8. Restriction analysis of the cloned fragments of the *hom3-10* gene. Lane 1: Lambda DNA (clind1ts857*S*am7) size markers (*Eco*RI/*Hin*dIII restriction fragments). Lane 2: *Eco*RI/*Pst*I digestion of the *Sma*I clone; the two fragments released are the 2.9 kb vector and the 1.3 kb insertion (the PCR amplified intact fragment). Lane 3: *Kpn*I digestion of the *Kpn*I clone; the two fragments released are the 2.9 kb vector and the 0.76 kb *Kpn*I fragment of the amplified gene. Lane 4: *Eco*RI/*Pst*I digestion of the *Sau*3A clone; the two fragments released are the 2.9 kb vector and the 0.36 kb *Sau*3A fragment of the amplified gene.

DISCUSSION

A. Mutagenicity of acridines

9-Aminoacridine is a frameshift mutagen in the yeast *Saccharomyces cerevisiae* (Table 4); it increased the reversion rate of the *hom3-10* frameshift allele significantly, although this mutagenic effect was apparent only at certain concentrations. The mutagenicity of 9-aminoacridine was also tested in two other frameshift alleles, *his4-38* and *leu2-3*. 9-Aminoacridine, at the same concentration which had a mutagenic effect on *hom3-10*, increased the reversion rates of *his4-38* and *leu2-3* as well.

As described previously, 9-aminoacridine binds reversibly, and in two different ways, to DNA (Peacocke, 1973): (1) A strong binding process, by intercalation between the stacked base pairs. Intercalation complexes are structures in which the planar ring system of a polycyclic molecule lies sandwiched between adjacent base pairs of the double helix. (2) A weak binding process, on the outside of the double helix, by electrostatic forces between the positively charged acridines and the negatively charged phosphate. Although the underlying mechanisms which lead from the intercalation of acridines into DNA to the production of a mutation are largely unknown at present, various models have been proposed to explain how acridine dyes cause frameshift mutations.

Brenner *et al.* (1961) suggested that intercalation between adjacent bases could induce miscopying errors which would add or delete single bases. Since the inhibition of T4 DNA synthesis by 5-fluorodeoxyuridine reduced proflavine mutagenesis only slightly (Drake, 1964), and since many frameshift mutations clearly add or delete two or more base pairs (Drake, 1966; Streisinger *et al.*, 1966), frameshift mutations caused by acridines are unlikely to arise as simple errors of replication. More recently, Freund *et al.* (1989) reported that Z-DNA-forming sequences are hot spots for spontaneous deletions, and all of the deletions involve an even number of base pairs. The chemical carcinogen 2-acetylaminofluorene induces, at high frequency, -2 frameshift mutations within sequences containing short stretches of alternating GCs. Alternating GC sequences are capable of adopting the Z conformation. Lerman (1963) suggested that acridines induce unequal crossing over by forcing mistakes in homologous pairing. The model suggests that correlations should exist between recombination and frameshift mutation, which would explain the strong response of T-even phages to acridines, compared with the response of phage lambda and bacteria, neither of which experience recombination very frequently (Drake, 1964).

Streisinger *et al.* (1966) surmised that frameshift mutations may be the consequence of mispairing errors during the repair of single-strand interruptions in a double-stranded DNA molecule. His slippage model proposed that local melting away from a nick, followed by misreannealing promoted by local base pair redundancy, would generate a frameshift mutation if the misannealed configuration were sealed up before the more probable, correctly annealed, configuration was reestablished. The role of acridines in this process is that they act to stabilize improbable mispairing configurations, thus increasing the chances of a repair process converting the mispaired configurations into heritable lesions. Each of these models assumes that the mutagenicity of acridines results from intercalation. There is evidence that intercalation by itself is not sufficient for the mutagenesis caused by acridines. Lerman (1964) reported a failure to observe any clear correlation between intercalating ability and mutagenic efficiency; some of the relatively strong intercalating agents failed to induce mutation.

Various cellular systems such as repair, replication, and recombination, which have been shown in general to modify and influence mutational yields in a variety of ways, may be involved in acridine mutagenesis. Newton *et al.* (1972) showed that the site of mutagenic action of 9-aminoacridine is associated with the DNA replication fork. They synchronized DNA synthesis in a culture of *Escherichia coli* cells and then treated the cells with 9-aminoacridine for 3- or 4-minute periods at various times after initiation of a new round of DNA replication. Extensive mutagenesis in a particular gene was seen only when the drug was added at about the time that a replication fork was passing through the gene. This correlation between acridine mutagenesis and the position of the replication fork suggests that the free polynucleotide end required by Streisinger's slippage model can be supplied either by the fork or by a gap between Okazaki fragments on the lagging strand. Streisinger and Owen (1985) showed that frequencies of proflavine-induced frameshift mutations increase dramatically as a function of the number of reiterated base pairs at each of two sites in the lysozyme gene of phage T4, and suggested that proflavine-induced mutagenesis may be due to mutagen-induced stuttering of the replication complex.

Acriflavine and acridine orange were also tested in the compartmentalization test. These two acridines are similar to 9-aminoacridine in structure but have different functional groups (Table 2). In 9-aminoacridine, there is an amino group at the "9" position of the acridine ring system. Acriflavine is a mixture of two acridines, proflavine and proflavine methochloride, both of which have amino groups at the "3" and the "6" positions, and proflavine methochloride has a methyl group at position "10". There are two dimethylamino groups in acridine orange, one at position "3" and the other at position "6". Just as with 9-aminoacridine, acriflavine and acridine orange are mutagenic to the frameshift allele, *hom3-10* (Table 8). The different functional groups could affect the binding properties of these acridines. At similar molar concentrations, acriflavine and acridine orange were more mutagenic when compared with 9-aminoacridine. None of these three acridines affect the reversion rates of the base substitution alleles, *arg4-17* and *his1-7* (Tables 6 and 8), therefore 9-aminoacridine, acriflavine, and acridine orange induce neither transitions nor transversions in mitotic yeast cells.

The complexity of the factors which govern the frameshift mutagenicity of different acridines may be a consequence of the problems which a repair enzyme encounters when it attempts to act upon a region of DNA in which the backbone has been elongated and distorted by intercalation of acridines. According to Streisinger's slippage model, if the mispaired region was to be stabilized by intercalation of acridines, the repair enzyme would have to act at almost the same site. The configuration of the broken ends of the DNA molecule which are to be joined, and the fit between the repair enzyme and the nearby portions of the DNA molecule, might then be rather subtly sensitive to the exact structure of the acridine which is present.

B. Antimutagenicity of 9-aminoacridine

9-Aminoacridine also has an antimutagenic effect (Table 7 and Fig. 6; Magni *et al.*, 1964). The antimutagenic effect is dose dependent; at lower concentrations, 9-aminoacridine is mutagenic and only at higher concentrations did it decrease the mutation rate for *hom3-10* to below the spontaneous level. Just as with the mutagenic effect, this antimutagenic effect is allele specific in that only the frameshift alleles were affected, and not the base substitution alleles. This antimutagenic effect on the frameshift alleles *hom3-10*, *his4-38*, and *leu2-3* was detected clearly at higher concentrations of 9-aminoacridine, but not with acriflavine and acridine orange. The observation that the reversion rates of base-substitution mutants *his1-7* and *arg4-17* remain unaffected with increasing concentrations of acridines is not consistent with the notion that revertant expression may have been inhibited by the acridines.

The interaction between the antimutagenic effect and the effect on cell division was studied, and at certain concentrations, 9-aminoacridine delayed cell division without a concurrent decrease in cell viability (Table 4 and Fig. 3). It is possible that this delay in cell division is involved in the expression of the antimutagenic effect. Both acriflavine and acridine orange delayed cell division with a concurrent decrease in cell viability. This decreased viability may be the reason that an antimutagenic effect was not observed with these two acridines. The delay in the cell generation time may be related to the binding properties of acridines. 9-Aminoacridine, acriflavine, and acridine orange exhibit both strong and weak binding modes (Lerman, 1964; Orgel and Brenner, 1961). The strong binding (*e.g*, intercalation) is observed at lower acridine/base pair ratios, whereas the weak binding, which occurs essentially on the outside of the DNA molecule, is observed at higher acridine/base pair ratios (Peacocke and Skerrett, 1956). The delay in cell division caused by these three acridines at higher concentrations could be related to the weak binding action. The stage in the cell cycle which is most likely to be affected by acridine binding on the outside of the DNA duplex would be DNA replication. The bound acridine molecules could affect the accessory protein factors of the replication machinery, remove or change the binding protein involved in the replication, or physically inhibit the progression of the replication fork.

9-Aminoacridine exhibits significant differences in its binding behavior in two respects. First, 9-aminoacridine molecules stack asymmetrically over one strand of a duplex (Young and Kallenbach, 1980). Proflavine, which is one component of acriflavine, and acridine orange intercalate symmetrically and overlap both strands of a duplex (Patel and Canuel, 1977). This symmetrical intercalation is attributable to the side chains of these two acridines (amino groups at positions "3" and "6" of proflavine, and dimethylamino groups at positions "3" and "6" of acridine orange). Second, as far as the preference of the binding site is concerned, *in vitro* experiments showed that 9-aminoacridine at lower concentrations binds preferentially at pGpC sites, whereas at higher concentrations this acridine binds preferentially at pApC or pGpT sites. Proflavine and acridine orange bind at pCpC/pGpG sites at both lower and higher concentrations (Patel and Canuel, 1977). The changing of the preferred binding sites of 9-aminoacridine at different concentrations could be related to the shift from mutagenic to antimutagenic effects at different concentrations.

Studies on mutators and antimutators in yeast have shown that the DNA repair systems play a very important role in generating the spontaneous mutations in this organism (Hastings *et al.*, 1976). There are two kinds of repair pathways, which can be referred to as accurate repair and mutagenic repair pathways. Mutators deficient in DNA repair enhance spontaneous mutation rates by channelling spontaneous lesions into mutagenic repair pathways. The delay in cell division caused by 9-aminoacridine may provide extra time for accurate repair to take place, or it may allow the accurate repair system to work at higher efficiency. The time factor thus would be enough to repair both 9-aminoacridine-induced damage and some spontaneous lesions.

Another property of this antimutagenic effect is that, after the effect appears, it does not increase with increasing concentrations of 9-aminoacridine and a plateau is observed (Fig. 6). At concentrations of 35, 40, 60, and 80 µg/ml, 9-aminoacridine decreased the spontaneous mutation rate of *hom3-10* by 2-fold (Table 7). This indicates that the antimutagenic effect may be an induced process in that either certain antimutagenic processes were turned on, or the mutagenic processes were turned off. An alternative explanation, which is discussed later, is the saturation of the mutagenic DNA repair system. Since the antimutagenic effect was observed only in the reversion of frameshift alleles, the repair pathway which involves frameshift mutagenesis should be considered as the mostly likely candidate for involvement in the antimutagenesis of 9-aminoacridine.

Genetic analysis of UV mutagenesis in yeast has led to the identification of more than 10 gene loci that are active in the production of UV-induced mutations (Lemontt, 1977). Although such loci appear to act within a single process dependent on *RAD6* function (Lawrence and Christensen, 1976), a number of them, in contrast to comparable genes in *Escherichia coli*, are concerned with the production of only certain types of UV-induced mutations. One such example is provided by the *rev2-1* mutation. This mutation, as well as mutations at the *REV1* and *REV3* loci, was isolated by Lemontt (1971) from strains of yeast in which the frequencies of *ARG*+ revertants (of the ocher allele *arg4-17*) induced by UV were reduced 4-fold. Subsequent observations have demonstrated that the *REV2* locus is concerned with the production of mutations that revert some, but not all, ocher mutations. The role of the *REV3* gene function in UV-induced mutagenesis in yeast has been examined by determining the reversion of 12 well-defined *cyc1* mutations in diploid strains homozygous for the *rev3-1* or *rev3-3* alleles (Lawrence and Christensen, 1979). The results imply that the *REV3* gene function is required for the production of a wide variety of mutational events. These events include both addition and deletion frameshifts. Since many UV-induced mutations in yeast affect the frequency of spontaneous mutation, it is believed that spontaneous lesions occur, and are repaired by systems which have many steps in common with the systems that repair UV-induced lesions (Hastings *et al.*, 1976). This was also observed with the *rev3* mutant; the antimutator allele of *rev3*, *rev3-1-5* (=*ant1*), was isolated (Quah *et al.*, 1980), and it reduces the reversion rate of *his1-7* in strain XV185-14C by 2-fold. This implies that *REV3* is involved in the mutagenic repair of spontaneous lesions, which results in base substitution mutations. As demonstrated by Lawrence and Christensen (1979), *REV3* gene function is required for the UV-induced frameshift mutations, and the *REV3* locus is also involved in the production of spontaneous frameshift mutations. Now Morrison *et al.* (1989) have shown that *REV3* encodes a DNA polymerase, which is perhaps the one involved in replication bypass of the lesion in the DNA.

9-Aminoacridine, at higher concentrations, could cause an antimutagenic effect by affecting the mutagenic repair system in which the *REV3* gene is involved. Since the antimutagenic effect does not increase with increasing concentrations of 9-aminoacridine, the mutagenic repair system being affected seems to become saturated. The antimutagenic effect of 9-aminoacridine is characterized by the more than 2-fold decrease in the mutation rate of *hom3-10*, from the spontaneous reversion rate of 0.8×10^{-8} to 0.36×10^{-8} (Table 7 and Fig. 6); a similar effect was evident with the frameshift alleles, *his4-38* and *leu2-3* (Table 7). With the antimutagenic effect of 9-aminoacridine, only one half of the spontaneous mutations remains, suggesting that the other half of the spontaneous lesions was repaired accurately. This implies that the systems which generate spontaneous frameshift mutations are not turned off completely. Therefore, there may be more than one repair pathway involved in frameshift mutagenesis.

Since 9-aminoacridine also showed a mutagenic effect at lower concentrations, the relationship between the mutagenic and antimutagenic processes should be considered. It is likely that the mutagenic and antimutagenic effects were caused by different systems affected by 9-aminoacridine. In fact, the antimutagenic effect could exist at lower concentrations as well, but because the mutagenic effect is much stronger than the antimutagenic effect, only the increase in the mutation rate was observed. If this were the case, the mutagenic effect of 9-aminoacridine would be stronger than what was actually observed, since the antimutagenic effect compensated for a portion of the mutagenic effect. This implies that the system causing the mutagenic effect of 9-aminoacridine was turned off or saturated completely at the concentrations where the antimutagenic effect was observed.

C. Suppression of frameshift mutations

Mutations which add or delete bases from DNA have drastic consequences because they shift the frame of the genetic message, which normally is read in groups of three bases, producing a sequence of altered amino acids in the protein or generating a premature stop codon. Frameshift suppressors in *Saccharomyces cerevisiae* have been studied in detail in the *his4* gene. A genetic analysis of several *his4* revertants showed that one class of ICR

170-induced frameshifts was suppressible by a frameshift suppressor (Riddle and Roth, 1970). This class can be divided into two groups of suppressible mutations, each group having its own suppressors. One type of suppressor suppresses the 4-base codon CCCN for proline and the other suppresses GGGN for glycine, in which N can be A, C, G, or U. Suppression of these +1 insertions could result from a forward mutation in a tRNA, which then inserts an amino acid in response to a 4-base codon. Support for this hypothesis came from experiments showing that this class of suppressor mutations affects the chromatographic behavior of prolyl- and glycyl-tRNAs (Riddle and Roth, 1972a, b). Direct confirmation of the involvement of tRNA in frameshift suppression was obtained by showing that strains of *Salmonella typhimurium* carrying the frameshift suppressor *sufD* produce a glycyl-tRNA with the nucleotide quadruplet CCCC at the anticodon position, instead of the triplet CCC found in the wild type (Riddle and Carbon, 1973). The addition of this extra base to the anticodon of the tRNA is presumed to permit the recognition of the 4-base codon GGGN and thereby the reading frame is restored. Culbertson *et al.* (1977) reported that 20 of the 21 ICR 170-induced *his4* mutants which revert with ICR 170 are suppressed by frameshift suppressors. The suppressors fall into two mutually exclusive groups based on their spectrum of suppression. Both groups of suppressors are dominant and fail to suppress nonsense, missense, and deletion type mutations (Culbertson *et al.*, 1977). Biochemical analysis showed that one group of suppressors has an altered glycyl-tRNA (Culbertson *et al.*, 1980).

The *hom3-10* allele is a frameshift mutation which involves a

+1 C insertion in a proline codon (Fig. 9). The mutant site is near the postulated end of the coding region, as determined from the wild-type sequence published by Rafalski and Falco (1988). The +1 C insertion shifts the reading frame, therefore the postulated stop codon is no longer within the frame. According to the shifted reading frame, the next stop codon is about 360 bp downstream (Fig. 9b). This indicates that the mutant protein will be 120 amino acids larger than the wild-type protein. This frameshift mutation can revert at the locus or it can be suppressed.

The locus events could take place both in upstream and downstream regions relative to the original fameshift mutation site. If the -1 deletion were to occur in the upstream region between base 1565 and the actual mutation site, a premature stop codon would not be generated (Fig. 9b). A -1 deletion in the downstream region could occur between the actual mutation site and the postulated stop codon, or any of the potential stop codons depicted in Fig. 9a. Also, the -1 deletion could occur within the actual mutation site or within any of the stop codons (Fig. 9a). These assumptions as to where the -1 deletion could occur are based on a computer exercise. Therefore, a -1 deletion (upstream or downstream) can correct the reading frame only, and the resulting protein will be different from the wild-type protein since the amino acid sequence between the actual mutation site and the -1 deletion site would be altered.

The suppression of the *hom3-10* frameshift mutation can involve a +1 base insertion in the anticodon region of a prolyl-tRNA and instead of CCA, CCCA will be read as a proline codon (Fig. 10a). The reading frame will be back to normal without there having been a change in the amino acid sequence at the locus and the protein will function as wild type.

The yeast strain XV195-6B has three frameshift alleles, which are *his4-38*, *leu2-3*, and *hom3-10*. *HIS*$^+$, *LEU*$^+$, and *HOM*$^+$ revertants can be selected on SC-omission media (SC-his, SC-leu, SC-met, and SC-thr) and the revertant colonies are replica plated to determine auxotrophic requirements. In this way, the apparent revertants which are actually still mutant but are being suppressed by frameshift suppressors can be identified by coreversion analysis. Several apparent revertants with a Hom$^+$His$^+$Leu$^+$ phenotype have been isolated (U.G.G. Hennig and K.S. Blonsky, unpublished data) and this indicates that the same suppressors which suppress the frameshift mutation in *hom3-10* can suppress *his4-38* and *leu2-3* simultaneously. The mutation in *hom3-10* is a +1 C insertion in a proline codon, whereas the mutations in *his4-38* and *leu2-3* are +1 G insertions in glycine codons (Fig. 10).

The mechanism by which a 4-base prolyl-tRNA suppresses the *hom3-10*, *his4-38*, and *leu2-3* mutations simultaneously could involve suppression at a target site other than the actual mutation. It has been reported that the frameshift suppressing tRNA of bacteria can suppress a mutation at a distance from the actual mutation site. A suppressing tRNA, *sufJ*, has been isolated from *Salmonella typhimurium* (Kohno *et al.*, 1983) and it is able to suppress a variety of +1 frameshift mutations (CCCU, GGGG, AAAA) by acting at a target site which is several bases away from the actual mutation site (Bossi and Roth, 1981). Further studies showed that a frameshift mutation which is not suppressible by *sufJ* can be converted into a suppressible one by a single base substitution upstream from the actual mutation to generate a target site for *sufJ* (Bossi and Roth, 1981). When the DNA sequences of *his4-38* and *leu2-3* were analyzed, it was apparent that the suppressing prolyl-tRNA potentially could act at a distance from the sites of the *his4-38* and *leu2-3* mutations (Fig. 10b, c). There is a proline codon immediately upstream from the mutant sites in both alleles, 3 bp away from the site of the *his4-38* mutation and 6 bp away from the site of the *leu2-3* mutation. The suppressing prolyl-tRNA could read 4-base proline codons at these two target sites (CCAA for *his4-38* and CCTA for *leu2-3*), thereby shifting the reading frame back to normal. Upon suppression at these distant sites, only one or two of the amino acids immediately upstream are altered; for *his4-38*, pro ser gly becomes pro val gly (Fig. 10b) and for *leu2-3*, pro lys trp gly becomes pro asp ala gly (Fig. 10c). It is assumed that these minor changes in the amino acids do not affect the function of the protein, therefore the wild-type phenotype was observed.

Bossi and Roth (1981) showed that codon recognition by frameshift suppressing tRNAs of *Salmonella typhimurium* does not require pairing with the base at position 4; the frameshift suppressor *sufJ* can read any 4-base codon with ACC in the first three positions. Gaber and Culbertson (1984) made the same observation in the yeast *Saccharomyces cerevisiae*. Nonetheless, interactions at position 4 may influence the efficiency of suppression. The recognition by the suppressing tRNA may not require base pairing at position 3. Since the frameshift suppressor reads the 5'-CCCA-3' proline codon in *hom3-10* (Fig. 10a), it is assumed that the anticodon of the suppressing tRNA is 3'-GGGU-5'. For *his4-38* and *leu2-3*, the codons being read are 5'-CCAA-3' and 5'-CCUA-3', respectively (Fig. 10b, c), thus base pairing at position 3 does not seem to be necessary in either case. This implies that base pairing at position 3 may not be essential for the recognition by the suppressing 4-base tRNA, although this base pairing at position 3 could affect the efficiency of suppression.

The efficiency of suppression was tested by crossing the triply suppressed mutants (Hom$^+$His$^+$Leu$^+$ phenotype) with strain XV195-10B, which has the identical genotype as XV195-6B but is of the opposite mating type (E.A. Savage, unpublished data). The diploid cells have the phenotype Hom$^+$His$^-$Leu$^-$, and all three alleles show 2:2 segregations in tetrad analysis. The results indicate that the suppression is more efficient for *hom3-10* than it is for *his4-38* and *leu2-3*, suggesting that pairing at position 3 enhances the efficiency of suppression.

This is a preliminary hypothesis for the mechanism by which the suppressing tRNA can suppress the frameshift mutation at a distance. In order to obtain more evidence to support this hypothesis, further genetic studies are needed. Sequencing the triply suppressed revertants and apparent locus revertants can identify the nature of these revertants. If the sequences of these revertants are identical to that of the *hom3-10* mutant, the reversion was not at the locus and the phenotype is due to frameshift suppressors. There are two possibilities for the frameshift suppression. First, a 4-base prolyl-tRNA suppresses all three mutations, and second, a 4-base prolyl-tRNA suppresses only *hom3-10* whereas a 4-base glycyl-tRNA suppresses *his4-38* and *leu2-3* at the actual mutation sites. The second possibility must be considered because revertants with a Hom$^+$His$^+$Leu$^+$ phenotype are infrequent enough (U.G.G. Hennig and K.S. Blonsky, personal communication) that the probability of two mutation events, though extremely unlikely, cannot be ruled out. These two alternatives can be distinguished by crossing the triply suppressed mutants with XV195-10B, and looking at the phenotype of the diploid cells. The Hom$^+$His$^-$Leu$^-$ phenotype would indicate that the suppression was caused by a suppressing prolyl-tRNA only, whereas the Hom$^+$His$^+$Leu$^+$ phenotype would indicate that the suppression was caused by 4-base prolyl- and glycyl-tRNAs. Another study to distinguish between these two possibilities could involve changing the potential target site for the suppression in *his4-38* and *leu2-3*, and checking for the continued suppressibility by the same 4-base prolyl-tRNA.

Fig. 9. The frameshift mutation in *hom3-10* and the consequence of the mutation. (a) The wild-type sequence of the distal part of the *HOM3* gene, and its 3' flanking region. Positions 1532 to 1606 are at the end of the coding region. After the postulated stop codon (**TAA**) at position 1607, there are several potential stop codons which are identified by an asterisk (•). (b) The mutation in *hom3-10* is an insertion of a **C** in a proline codon. This insertion shifts the reading frame so that the next stop codon is at position 1946, extending the length of the mutant protein by 120 amino acids.

a. *HOM*$^{+}$

```
1532 AAG TTA GTC GTA GAT TTA ATA TCT ACT TCT GAA GTT CAT GTT TCG ATG GCT TTG CCA TTC
     lys leu val val asp leu ile ser thr ser glu val his val ser met ala leu pro phe

1592 CAG ATG CAG ACT CAT TAA AAT CTC TGA GAC AAG CTG AGG AAA AAT TGA GAA TTT TAG GTT
     gln met gln thr his  *  asn leu  *  asp lys leu arg lys asn  *  glu phe  *  val

1652 CTG TTG ATA TCA CAA AGA AGT TGT CTA TTG TTT CAT TAG TTG GTA AAC ATA TGA AAC AAT
     leu leu ile ser gln arg ser cys leu leu phe his  *  leu val asn ile  *  asn asn

1712 ACA TCG GCA TTG CTG GTA CCA TGT TTA CTA CTC TTG CTG AAG AAG GCA TCA ACA TTG AAA
     thr ser ala leu leu val pro cys leu leu leu leu leu lys lys ala ser thr leu lys

1772 TGA TTT CTC AAG GGG CAA ATG AAA TAA ACA TAT CCT GCG TTA TCA ATG AAT CTG ACT CCA
      *  phe leu lys gly gln met lys  *  thr tyr pro ala leu ser met asn leu thr pro

1832 TAA AAG CGC TAC AAT GTA TTC ATG CCA AGT TAC TAA GTG AGC GGA CAA ATA CTT CAA ACC
      *  lys arg tyr asn val phe met pro ser tyr  *  val ser gly gln ile leu gln thr

1892 AAT TTG AAC ATG CCA TTG ATG AAC GTT TAG AAC AAT TGA AAA GAC TTG GAA TTT AAA
     asn leu asn met pro leu met asn val  *  asn asn  *  lys asp leu glu phe lys
```

b. *hom3-10*

```
                                                       Site of the +1 C insertion
                                                                      ↓
1532 AAG TTA GTC GTA GAT TTA ATA TCT ACT TAC GAA GTT CAT GTT TCG ATG GCT TTG CCC ATT
     lys leu val val asp leu ile ser thr tyr glu val his val ser met ala leu pro ile

1592 CCA GAT GCA GAC TCA TTA AAA TCT CTG AGA CAA GCT GAG GAA AAA TTG AGA ATT TTA GGT
     pro asp ala asp ser leu lys ser leu arg gln ala glu glu lys leu arg ile leu gly

1652 TCT GTT GAT ATC ACA AAG AAG TTG TCT ATT GTT TCA TTA GTT GGT AAA CAT ATG AAA CAA
     ser val asp ile thr lys lys leu ser ile val ser leu val gly lys his met lys gln

1712 TAC ATC GGC ATT GCT GGT ACC ATG TTT ACT ACT CTT GCT GAA GAA GGC ATC AAC ATT GAA
     tyr ile gly ile ala gly thr met phe thr thr leu ala glu glu gly ile asn ile glu

1772 ATG ATT TCT CAA GGG GCA AAT GAA ATA AAC ATA TCC TGC GTT ATC AAT GAA TCT GAC TCC
     met ile ser gln gly ala asn glu ile asn ile ser cys val ile asn glu ser asp ser

1832 ATA AAA GCG CTA CAA TGT ATT CAT GCC AAG TTA CTA AGT GAG CGG ACA AAT ACT TCA AAC
     ile lys ala leu gln cys ile his ala lys leu leu ser glu arg thr asn thr ser asn

1892 CAA TTT GAA CAT GCC ATT GAT GAA CGT TTA GAA CAA TTG AAA AGA CTT GGA ATT TAA
     gln phe glu his ala ile asp glu arg leu glu gln leu lys arg leu gly ile  *
```

Fig. 10. Suppression of the frameshift mutations in *hom3-10*, *his4-38*, and *leu2-3*. The bases in outline type identify the sites of the insertions (+1 C in a proline codon in *hom3-10*, +1 G in a glycine codon in both *his4-38* and *leu2-3*). The 4-base codons in bold type are the target sites for the frameshift suppressing prolyl-tRNA. These suppressing prolyl-tRNAs suppress the frameshift mutations in *his4-38* and *leu2-3* at a distance.

a.

	1580					
HOM3⁺	GCT	TTG	CCA	TTC	CAG	
	ala	leu	pro	phe	glu	
hom3-10	GCT	TTG	CCCA	TTC	CAG	
	ala	leu	pro -	phe	glu	
						1946
Shifted reading frame	GCT	TTG	CCC	ATT	CCA	..........TAA
	ala	leu	pro	ile	pro	................*
Suppressed reading frame	GCT	TTG	**CCCA**	TTC	CAG	
	ala	leu	pro	phe	glu	

b.

	166					
HIS4⁺	TTG	CCA	AGT	GGT	AAA	TTC
	leu	pro	ser	gly	lys	phe
his4-38	TTG	CCA	AGT	GGGT	AAA	TTC
	leu	pro	ser	gly	lys	phe
Shifted reading frame	TTG	CCA	AGT	GGG	TAA	ATT
	leu	pro	ser	gly	*	
Suppressed reading frame	TTG	**CCAA**	GTG	GGT	AAA	TTC
	leu	pro	val	gly	lys	phe

c.

	235							
LEU2⁺	GGT	CCT	AAA	TGG	GGT	ACC	GGT	AGT
	gly	pro	lys	trp	gly	thr	gly	ser
leu2-3	GGT	CCT	AAA	TGG	GGGT	ACC	GGT	AGT
	gly	pro	lys	trp	gly	thr	gly	ser
Shifted reading frame	GGT	CCT	AAA	TGG	GGG	TAC	CGG	TAG
	gly	pro	lys	try	gly	tyr	arg	*
Suppressed reading frame	GGT	**CCTA**	AAT	GGG	GGT	ACC	GGT	AGT
	gly	pro	asp	ala	gly	thr	gly	ser

BIBLIOGRAPHY

Adachi, A. (1969). Effect of acriflavine and 4-nitroquinoline*N*-oxide on *Saccharomyces cerevisiae*. *Biological Journal (Nara Women's University)* **18:** 12-14.

Arakatsu, Y. (1971). Action of acriflavine on growth and mutation in yeast. I. Nitrogen sources affecting the mutation induction. *Mutation Research* **12:** 235-248.

Avers, C.J., and C.D. Dryfuss (1965). Influence of added nucleosides on acriflavine induction of petite mutants in baker's yeast. *Nature* **206:** 850.

Bien, M., and B. Konrad (1972). Sensitivity of *Saccharomyces cerevisiae* in various phases of the life cycle to induction by acriflavine. III. Induction of mutants during sporulation. *Acta Microbiologica Polonica, Series A, Microbiologia Generalis* **4:** 99-106.

Bossi, L., and J.R. Roth (1981). Four-base codons ACCA, ACCU, and ACCC are recognized by frameshift suppressor *sufJ*. *Cell* **25:** 489-496.

Brenner, S., L. Barnett, F.H.C. Crick, and A. Orgel (1961). The theory of mutagenesis. *Journal of Molecular Biology* **3:** 121-124.

Brown, B.R., W.J. Firth III, and L.W. Yielding (1980). Acridine structure correlated with mutagenic activity in Salmonella. *Mutation Research* **72:** 373-388.

Cox, B.S. (1965). ψ, a cytoplasmic suppressor of super-suppressors in yeast. *Heredity* **20:** 505-521.

Cox, B.S., M.F. Tuite, and C.S. McLaughlin (1988). The ψ factor of yeast: A problem in inheritance. *Yeast* **4:** 159-178.

Culbertson, M.R., L. Charnas, M.T. Johnson, and G.R. Fink (1977). Frameshifts and frameshift suppressors in *Saccharomyces cerevisiae*. *Genetics* **86:** 745-764.

Culbertson, M.R., K.M. Underbrink, and G.R. Fink (1980). Frameshift suppressors in *Saccharomyces cerevisiae*. II. Genetic properties of group II suppressors. *Genetics* **95:** 833-853.

Davies, P.J., W.E. Evans, and J.M. Parry (1975). Mitotic recombination induced by chemical and physical agents in the yeast *Saccharomyces cerevisiae*. *Mutation Research* **29:** 301-314.

DeMars, R.I. (1953). Chemical mutagenesis in bacteriophage T2. *Nature* **172:** 964.

Demerec, M., G. Bertani, and J. Flint (1951). A survey of chemicals for mutagenic action in *E. coli*. *American Naturalist* **85:** 119-136.

Dickie, P., R. Morgan, D.G. Scraba, and R.C. von Borstel (1985). The binding of the anthelmintic pyrvinium cation to deoxyribonucleic acid *in vitro*. *Molecular Pharmacology* **29:** 427-435.

Donahue, T.F., P.J. Farabaugh, and G.R. Fink (1981). Suppressible four-base glycine and proline codons in yeast. *Science* **212:** 455-457.

Drake, J.W. (1964). Studies on the induction of mutations in bacteriophage T4 by ultraviolet irradiation and by proflavine. *Journal of Cellular and Comparative Physiology* **64 (Supplement 1):** 19-31.

Drake, J.W. (1966). Heteroduplex heterozygotes in bacteriophage T4 involving mutations of various dimensions. *Proceedings of the National Academy of Sciences (U.S.A.)* **55:** 506-512.

Drake, J.W., and E.F. Allen (1968). Antimutagenic DNA polymerase of bacteriophage T4. *Cold Spring Harbor Symposia on Quantitative Biology* **33:** 339-344.

Edelman, I. (1987). Recessive lethal translational suppressor codon recognition in *Saccharomyces cerevisiae*. Ph.D. Thesis. Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706 U.S.A., p. 150.

Fahrig, R. (1970). Acridine-induced mitotic gene conversion (paramutation) in *Saccharomyces cerevisiae:* The effect of two different modes of binding to DNA. *Mutation Research* **10:** 509-514.

Fink, G.R. (1964). Gene-enzyme relations in histidine biosynthesis in yeast. *Science* **146:** 525-527.

Fink, G.R., and C. Styles (1974). Gene conversion of deletions in the *HIS4* region of yeast. *Genetics* **77:** 231-244.

Freund, A.E., M. Bichara, and R.P.P. Fuchs (1989). Z-DNA-forming sequences are spontaneous deletion hot spots. *Proceedings of the National Academy of Sciences (U.S.A.)* **86:** 7456-7469.

Gaber, R.F., and M.R. Culbertson (1984). Codon recognition during frameshift suppression in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **4:** 2052-2061.

Gesteland, R.F., M. Wolfner, P. Grisafi, G. Fink, D. Botstein, and J.R. Roth (1976). Yeast suppressors of UAA and UAG nonsense codons work efficiently *in vitro* via tRNA. *Cell* **7:** 381-390.

Gilmore, R.A., J.W. Stewart, and F. Sherman (1971). Amino acid replacements resulting from super-suppression of nonsense mutants of iso-1-cytochrome *c* from yeast. *Journal of Molecular Biology* **61:** 157-173.

Goodman, H.M., M.V. Olson, and B.D. Hall (1977). Nucleotide sequence of a mutant eukaryotic gene: The yeast tyrosine-inserting ochre suppressor *SUP4-o*. *Proceedings of the National Academy of Sciences (U.S.A.)* **74:** 5453-5457.

Gottlieb, D.J.C., and R.C. von Borstel (1976). Mutators in *Saccharomyces cerevisiae: mut1-1, mut1-2* and *mut2-1*. *Genetics* **83:** 655-666.

Hartman, P.E., H. Berger, and Z. Hartman (1973). Comparison of hycanthone ("Etrenol"), some hycanthone analogs, myxin, and 4-nitroquinoline-1-oxide as frameshift mutagens. *Journal of Pharmacology and Experimental Therapeutics* **186:** 390-398.

Hartman, P.E., K. Levine, Z. Hartman, and H. Berger (1971). Hycanthone: A frameshift mutagen. *Science* **172:** 1058-1060.

Hastings, P.J., S-K. Quah, and R.C. von Borstel (1976). Spontaneous mutation by mutagenic repair of spontaneous lesions in DNA. *Nature* **264:** 719-722.

Hawthorne, D.C., and J. Friis (1964). Osmotic-remedial mutants. A new classification for nutritional mutants in yeast. *Genetics* **50:** 829-839.

Hennig, U.G.G. (1984). Genetic toxicology of pyrvinium pamoate. Ph.D. Thesis. Department of Genetics and Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta T6G 2E1 Canada, p. 8.

Hennig, U.G.G., L.G. Chatten, R.A. Pon, and R.C. von Borstel (1987). The detection of chemical impurities by high pressure liquid chromatography and the genetic activity of medical grades of pyrvinium pamoate in *Saccharomyces cerevisiae* and *Salmonella typhimurium*. *Archives of Toxicology* **60:** 278-286.

Hershfield, M.S., and N.G. Nossal (1973). *In vitro* characterization of a mutator T4 DNA polymerase. *Genetics* **73 (Supplement):** 131-136.

Heslot, H., C. Louis, and A. Goffeau (1970). Segregational respiratory deficient mutants of a "petite negative" yeast *Schizosaccharomyces pombe* 972 h^-. *Journal of Bacteriology* **104:** 482-491.

Kada, T. (1982). Mechanisms and genetic implications of environmental antimutagenesis. *In* "Environmental Mutagens and Carcinogens". T. Sugimura, S. Kondo, and H. Takebe (Editors). University of Tokyo Press (Tokyo) and Alan R. Liss, Incorporated (New York), pp. 355-359.

Keesey, J., R. Bigelis, and G. Fink (1979). The product of the *his4* gene cluster in *Saccharomyces cerevisiae*: A trifunctional polypeptide. *Journal of Biological Chemistry* **254:** 7427-7433.

Kim, H-S., and O. Smithies (1988). Recombinant fragment assay for gene targeting based on the polymerase chain reaction. *Nucleic Acids Research* **16:** 8887-8903.

Kohno, T., L. Bossi, and J.R. Roth (1983). New suppressors of frameshift mutations in *Salmonella typhimurium*. *Genetics* **103:** 23-29.

Krieg, D.R. (1963). Ethyl methanesulfonate-induced reversion of bacteriophage T4rII mutants. *Genetics* **48:** 562-580.

Lawrence, C.W., and R.B. Christensen (1976). UV mutagenesis in radiation-sensitive strains of yeast. *Genetics* **82:** 207-232.

Lawrence, C.W., and R.B. Christensen (1979). Ultraviolet-induced reversion of *cyc1* alleles in radiation-sensitive strains of yeast. III. *rev3* mutant strains. *Genetics* **92:** 397-408.

Lax, C., and S. Fogel (1978). Novel interallelic complementation at the *his1* locus of yeast. *Genetics* **90:** 501-516.

Lemontt, J.F. (1971). Mutants of yeast defective in mutation induced by ultraviolet light. *Genetics* **68:** 21-23.

Lemontt, J.F. (1977). Pathways of ultraviolet mutability in *Saccharomyces cerevisiae*. III. Genetic analysis and properties of mutants resistant to ultraviolet induced forward mutation. *Mutation Research* **43:** 179-204.

Lerman, L.S. (1961). Structural considerations in the interaction of DNA and acridines. *Journal of Molecular Biology* **3:** 18-30.

Lerman, L.S. (1963). The structure of the DNA-acridine complex. *Proceedings of the National Academy of Sciences (U.S.A.)* **49:** 94-102.

Lerman, L.S. (1964). Acridine mutagens and DNA structure. *Journal of Cellular and Comparative Physiology* **64:** 1-18.

Luria, S.E., and M. Delbrück (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28:** 491-511.

Magni, G.E. (1963). The origin of spontaneous mutations during meiosis. *Proceedings of the National Academy of Sciences (U.S.A.)* **50:** 975-980.

Magni, G.E., and P.P. Puglisi (1966). Mutagenesis of super-suppressors in yeast. *Cold Spring Harbor Symposia on Quantitative Biology* **31:** 699-704.

Magni, G.E., and R.C. von Borstel (1962). Different rates of spontaneous mutation during mitosis and meiosis in yeast. *Genetics* **47:** 1097-1108.

Magni, G.E., R.C. von Borstel, and S. Sora (1964). Mutagenic action during meiosis and antimutagenic action during mitosis by 5-aminoacridine in yeast. *Mutation Research* **1:** 227-230.

Mahler, H.R. (1973). Structural requirements for mitochondrial mutagenesis. *Journal of Supramolecular Structure* **1**: 449-460.

Malling, H.V., and F.J. de Serres (1968). Identification of genetic alterations induced by ethylmethanesulfonate in *Neurospora crassa*. *Mutation Research* **6**: 181-193.

Maniatis, T., E.F. Fritsch, and J. Sambrook (1982). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 545 pages.

McLarg, D.O., and W.D. Bowers (1968). Mitochondrial changes accompanying acriflavine-induced petite mutation in *Saccharomyces fragilis*. *Journal of Ultrastructure Research* **25**: 37-45.

Meadows, M.G. (1973). Mutagenic action of indazole analogs on yeast. Ph.D. Thesis. Department of Genetics, University of Alberta, Edmonton, Alberta T6G 2E9 Canada, p. 11.

Meadows, M.G., S-K. Quah, and R.C. von Borstel (1973). Mutagenic action of hycanthone and IA-4 on yeast. *Journal of Pharmacology and Experimental Therapeutics* **187**: 444-450.

Meyer, R.R., G.S. Probst, and S. Keller (1972). RNA synthesis by isolated mammalian mitochondria and nuclei: Effects of ethidium bromide and acriflavine. *Archives of Biochemistry and Biophysics* **148**: 425-430.

Morrison, A., R. Christensen, J. Alley, A.K. Beck, E.G. Bernstine, J.F. Lemontt, and C.W. Lawrence (1989). *REV3*, a *Saccharomyces cerevisiae* gene whose function is required for induced mutagenesis, is predicted to encode a nonessential DNA polymerase. *Journal of Bacteriology* **171**: 5659-5667.

Mortimer, R.K., and D. Schild (1981). Genetic map of *Saccharomyces cerevisiae*. *In* "The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance". J.N. Strathern, E.W. Jones, and J.R. Broach (Editors). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 645-651.

Newton, A., D. Masys, E. Leonardi, and D. Wygal (1972). Association of induced frameshift mutagenesis and DNA replication in *E. coli.* *Nature (New Biology)* **236:** 19-22.

Orgel, A., and S. Brenner (1961). Mutagenesis of bacteriophage T4 by acridines. *Journal of Molecular Biology* **3:** 762-768.

Patel, D.J., and L.L. Canuel (1977). Sequence specificity of mutagen-nucleic acid complex in solution: Interaction and mutagen-base pair overlap geometries for proflavine binding to dC-dC-dG-dG and dG-dG-dC-dC self-complementary duplexes. *Proceedings of the National Academy of Sciences (U.S.A.)* **74:** 2524-2531.

Peacocke, A.R. (1973). The interaction of acridines with nucleic acids. *In* "Chemistry of Heterocyclic Compounds". R.M. Acheson (Editor). Springer, New York, pp. 723-757.

Peacocke, A.R., and J.N.H. Skerrett (1956). The interaction of aminoacridines with nucleic acids. *Transactions of the Faraday Society* **52:** 261-279.

Pritchard, N.J., A. Blake, and A.R. Peacocke (1966). Modified intercalation model for the interaction of amino acridine and DNA. *Nature* **212:** 1360-1361.

Puglisi, P.P. (1966). Mutagenic and antimutagenic effects of acridine salts in yeast. *Genetics* **54:** 315-322.

Quah, S-K., R.C. von Borstel, and P.J. Hastings (1980). The origin of spontaneous mutation in *Saccharomyces cerevisiae*. *Genetics* **96:** 819-839.

Rafalski, J.A., and S.C. Falco (1988). Structure of the yeast *HOM3* gene which encodes aspartokinase. *Journal of Biological Chemistry* **263:** 2146-2151.

Riddle, D., and J. Carbon (1973). A nucleotide addition in the anticodon of a glycine tRNA. *Nature (New Biology)* **242:** 230-234.

Riddle, D., and J. Roth (1970). Suppressors of frameshift mutations in *Salmonella typhimurium*. *Journal of Molecular Biology* **54:** 131-144.

Riddle, D., and J. Roth (1972a). Frameshift suppressors. II. Genetic mapping and dominance studies. *Journal of Molecular Biology* **66:** 483-493.

Riddle, D., and J. Roth (1972b). Frameshift suppressors. III. Effects of suppressor mutations on tRNA. *Journal of Molecular Biology* **66:** 495-506.

Saiki, R.K., D.H. Gelfand, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich (1988). Primer-directed enzymatic amplification of DNA with a thermostable polymerase. *Science* **239:** 487-491.

Shahin, M.M., and R.C. von Borstel (1978). Comparisons of mutation induction in reversion systems of *Saccharomyces cerevisiae* and *Salmonella typhimurium*. *Mutation Research* **53:** 1-10.

Sherman, F. (1982). Suppression in the yeast *Saccharomyces cerevisiae*. *In* "The Molecular Biology of the yeast Saccharomyces: Metabolism and Gene Expression". J.N. Strathern, E.W. Jones, and J.R. Broach (Editors). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 463-486.

Sherman, F., G.R. Fink, and J.B. Hicks (1986). Techniques and protocols: Yeast DNA isolations. *In* "Laboratory Course Manual for Methods in Yeast Genetics". Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 125-126.

Smirnov, M.N., V.N. Smirnov, E.I. Budowsky, S.G. Inge-Vechtomov, and N.G. Serebrjakov (1967). Red pigment of adenine-deficient yeast *Saccharomyces cerevisiae*. *Biochemical and Biophysical Research Communications* **27:** 299-304.

Speyer, J.F. (1965). Mutagenic DNA polymerase. *Biochemical and Biophysical Research Communications* **21:** 6-8.

Speyer, J.F., J.D. Karam, and A.B. Lenny (1966). *Cold Spring Harbor Symposia on Quantitative Biology* **31:** 693-697.

Streisinger, G.Y., Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi, and M. Inouye (1966). Frameshift mutations and the genetic code. *Cold Spring Harbor Symposia on Quantitative Biology* **31:** 77-84.

Streisinger, G., and J. Owen (1985). Mechanisms of spontaneous and induced frameshift mutation in bacteriophage T4. *Genetics* **109:** 633-659.

Ullmann, A., F. Jacob, and J. Monod (1967). Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Escherichia coli*. *Journal of Molecular Biology* **24:** 339-343.

von Borstel, R.C. (1978). Measuring spontaneous mutation rates in yeast. *In* "Methods in Cell Biology", Vol. 20. D.M. Prescott (Editor), Academic Press, New York, pp. 1-24.

von Borstel, R.C. (1981). The yeast *Saccharomyces cerevisiae:* An assay organism for environmental mutagens. *In* "Short-Term Tests for Chemical Carcinogens". H.F. Stich and R.H.C. San (Editors). Spring-Verlag, New York, Chapter15, pp. 161-174.

von Borstel, R.C., K.T. Cain, and C.M. Steinberg (1971). Inheritance of spontaneous mutability in yeast. *Genetics* **69:** 17-27.

von Borstel, R.C., S-K. Quah, C.M. Steinberg, F. Flury, and D.J.C. Gottlieb (1973). Mutants of yeast with enhanced spontaneous mutation rates. *Genetics* **(Supplement)** **73:** 141-151.

von Borstel, R.C., E.A. Savage, P.O. Andersson, and U.G.G. Hennig (1986). A frameshift detection assay in the yeast *Saccharomyces cerevisiae* which detects base-pair additions and deletions as separable events. *Environmental Mutagenesis* **8(s6):** 88 (Abstract 236).

Watson, J.D., and F.H.C. Crick (1953). The structure of DNA. *Cold Spring Harbor Symposia on Quantitative Biology* **18:** 123-131.

Webb, R.B., and H.E. Kubitschek (1963). Mutagenic and antimutagenic effects of acridine orange in *Escherichia coli.* *Biochemical and Biophysical Research Communications* **13:** 90-94.

Witkin, E.M. (1947). Mutations in *E. coli* induced by chemical agents. *Cold Spring Harbor Symposia on Quantitative Biology* **12:** 256-269.

Young, P.R., and N.R. Kallenbach (1980). Site exclusion and sequence specificity in binding of 9-aminoacridine to the deoxytetranucleotide dpApGpCpTp. *Proceedings of the National Academy of Sciences (U.S.A.)* **77:** 6453-6457.